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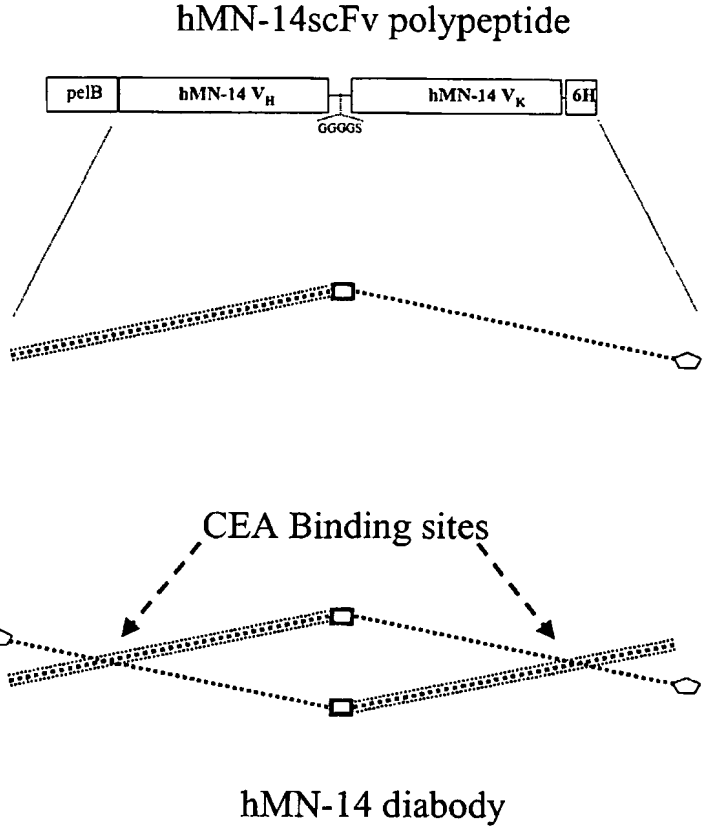
(54) Title: DIRECT TARGETING BINDING PROTEINS



(57) Abstract: The present invention relates to multivalent, monospecific binding proteins. These binding proteins comprise two or more binding sites, where each binding site specifically binds to the same type of target cell, and preferably with the same antigen on such a target cell. The present invention further relates to compositions of monospecific diabodies, triabodies, and tetrabodies, and to recombinant vectors useful for the expression of these functional binding proteins in a microbial host. Also provided are methods of using invention compositions in the treatment and/or diagnosis of tumors.

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DIRECT TARGETING BINDING PROTEINS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Applications 60/328,835, filed Oct. 15, 2001, 60/341,881 filed Dec. 21, 2001, 60/345,641 filed Jan. 8, 2002 and 60/404,919, filed Aug. 22, 2002, the contents of each of which are hereby incorporated herein in their entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to multivalent, monospecific binding proteins. In particular, the present invention relates to compositions of monospecific diabodies, triabodies, and tetrabodies and methods of use thereof, and to recombinant vectors useful for the expression of these functional binding proteins in a microbial host.

BACKGROUND OF THE INVENTION

[0003] The following description is provided to assist the understanding of the reader. None of the information provided or references cited is admitted to be prior art to the present invention.

[0004] Man-made binding proteins, in particular, monoclonal antibodies and engineered antibodies or antibody fragments, have been tested widely and have been shown to be of value in the detection and treatment of various human disorders, including cancers, autoimmune diseases, infectious diseases, inflammatory diseases and cardiovascular diseases (Filpula and McGuire, *Exp. Opin. Ther. Patents* 9:231-245 (1999)). For example, antibodies labeled with radioactive isotopes have been used to visualize tumors using detectors available in the art, following their injection into a patient. The clinical utility of an antibody or an antibody-derived agent is primarily dependent on its ability to specifically bind to a target antigen. Selectivity is valuable for the effective delivery of a diagnostic or a therapeutic agent (such as drugs, toxins,

cytokines, hormones, growth factors, conjugates, radionuclides, or metals) to a target location for the detection and/or treatment phases of a human disorder, particularly if the diagnostic or therapeutic agent is toxic to normal tissue in the body.

[0005] The potential limitations of antibody systems are known in the art (see, *e.g.*, Goldenberg, *Am. J. Med.* **94**:297-312 (1993)). Important parameters in detection and treatment techniques include, for example, the amount of the injected dose specifically localized at the site(s) where cells containing the target antigen are present and the uptake ratio, *i.e.* the ratio of the amount of specifically bound antibody to that of the free antibody present in surrounding normal tissues (as detected by radioactivity). When an antibody is injected into the blood stream, it passes through a number of physiological compartments as it is metabolized and excreted. Optimally, the antibody should be able to locate and bind to the target cell antigen while passing through the rest of the body. Factors that control antigen targeting include, for example, the location and size of the antigen, antigen density, antigen accessibility, the cellular composition of the target tissue, and the pharmacokinetics of the targeting antibodies. Other factors that specifically affect tumor targeting by antibodies include the expression levels of the target antigen, both in tumor and normal tissues, and bone marrow toxicity resulting from slow blood-clearance of radiolabeled antibodies.

[0006] The amount of targeting antibodies accreted by targeted tumor cells is influenced by vascularization of the tumor and barriers to antibody penetration of tumors, as well as intratumoral pressure. Non-specific uptake by non-target organs (such as the liver, kidneys or bone marrow) is another potential limitation of the technique, especially for radioimmunotherapy, where irradiation of the bone marrow often causes dose-limiting toxicity.

[0007] An approach referred to as direct targeting, is designed to target tumor antigens using antibodies carrying a diagnostic or therapeutic radioisotope. The direct targeting approach requires a radiolabeled anti-tumor monospecific antibody that specifically recognizes a target antigen located on or within the tumor. The technique

generally involves injecting the labeled monospecific antibody into the patient and allowing the antibody to localize to the tumor to obtain diagnostic or therapeutic benefits, while unbound antibody clears the body. However, the radiolabeled antibody does not form a very stable complex with the target antigen, and therefore, does not remain at the tumor site for a long period of time.

[0008] Thus, there remains a need in the art for compositions of multivalent, monospecific antibodies and methods of producing such antibodies using recombinant DNA technology for use in a direct targeting system. Specifically, there remains a need for an antibody that exhibits enhanced antibody uptake and binding to target antigens, leaving less free antibody in the circulation, and optimal protection of normal tissues and cells from toxic agents complexed with the antibody.

SUMMARY OF THE INVENTION

[0009] The present invention relates to multivalent, monospecific binding proteins. These binding proteins comprise two or more binding sites, where each binding site specifically binds to the same type of target cell, and preferably with the same antigen on such a target cell. The present invention further relates to compositions of monospecific diabodies, triabodies, and tetrabodies, and to recombinant vectors useful for the expression of these functional binding proteins in a microbial host. Also provided are methods of using invention compositions in the treatment and/or diagnosis of tumors. It is a specific object of the present invention to provide antibodies that exhibit enhanced antibody uptake and binding to target antigens, for use in the diagnosis and treatment of tumors.

[0010] According to one aspect of the present invention there are provided multivalent, monospecific binding proteins which have two or more binding sites specific for the same target antigen. Each binding site is formed by the association of two or more single chain Fv (scFv) fragments, and each scFv comprises at least 2 variable domains derived from a humanized or human monoclonal antibody. In various

alternative preferred embodiments, the multivalent, monospecific binding protein may be a monospecific diabody, a monospecific triabody, or a monospecific tetrabody. In preferred embodiments, the humanized or human monoclonal antibody is specific for a tumor-associated antigen, most preferably the carcinoembryonic antigen (CEA).

[0011] According to another aspect of the present invention, the multivalent, monospecific binding protein may also contain a diagnostic agent, a therapeutic agent, and/or combinations of two or more of such agents. In various embodiments, the diagnostic agent may be a conjugate, a radionuclide, a metal, a contrast agent, a tracking agent, a detection agents, or a combination thereof. In various embodiments, the therapeutic agent may be a radionuclide, a chemotherapeutic drug, a cytokine, a hormone, a growth factor, a toxin, an immunomodulator, or a combination thereof.

[0012] According to yet another aspect of the present invention there are provided expression vectors comprising nucleotide sequences that encode the various multivalent, monospecific binding proteins, as well as host cells that have been transformed with these expression vectors for the production of the binding proteins.

[0013] The present invention further provides methods of diagnosing the presence of a tumor and methods of treating a tumor using invention binding proteins. The binding proteins of the present invention also serve as an effective means of delivering one or more diagnostic agent, one or more therapeutic agent, or a combination of two or more thereof to a tumor in a subject; and may be conveniently provided in a kit for therapeutic and/or diagnostic use for practitioners.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] *Figure 1* is a schematic representation of the hMN-14scFv polypeptide synthesized in *E. coli* from the hMN-14-scFv-L5 expression plasmid, and the formation of a hMN-14 diabody. The nucleic acid construct encoding the unprocessed polypeptide contains sequences encoding the pelB signal peptide, the hMN-14V_H and hMN-14V_K coding sequences coupled by a 5 amino acid linker, and a carboxyl terminal

histidine affinity tag. The figure also shows a stick figure drawing of the mature polypeptide following proteolytic removal of the pelB signal peptide, and a stick figure drawing of a hMN-14 diabody, including CEA binding sites.

[0015] *Figure 2* collectively shows the results of size-exclusion high performance liquid chromatography (HPLC) analysis of hMN-14 diabody purification. Figure 2A is the HPLC elution profile of IMAC-purified hMN-14 diabody. The HPLC elution peaks of hMN-14 diabody in Figures 2A and 2B are identified with an arrow. Figure 2B is the HPLC elution profile of hMN-14 diabody purified by WI2 anti-idiotype affinity chromatography. The *9.75 indicated on the x-axis of Figure B is the HPLC retention time (9.75 minutes) of control hMN-14-Fab'-S-NEM (MW ~50 kDa).

[0016] *Figure 3* collectively shows the results of protein analysis of the hMN-14scFv polypeptide. Figure 3A is a reducing SDS-PAGE gel stained with Coomassie blue illustrating the purity of the hMN-14 diabody samples following IMAC purification and WI2 anti-idiotype affinity purification. The positions of the molecular weight standards and the hMN-14scFv polypeptide are indicated with arrows. Figure 3B is an isoelectric focusing (IEF) gel. The positions of pI standards and hMN-14scFv polypeptide are indicated with arrows. Lane 1 of Figure 3B contains the hMN-14 Fab'-S-NEM used as a standard. Lane 2 of the same figure contains the WI2 purified hMN-14 diabody. Lane 3 contains the unbound flow-through fraction from the WI2 affinity column, which indicated that the hMN-14scFv diabody is effectively purified by this process.

[0017] *Figure 4* shows the level of ^{131}I-hMN-14 diabody over the first 96 hours following injection of the diabody as monitored in tumor and blood samples. The amount of ^{131}I-hMN-14 diabody, measured as the percentage of the injected dose per gram of tissue (%ID/g), is plotted against time. Solid squares mark the data points for tumor samples and open boxes mark those of blood samples.

[0018] *Figure 5* shows the biodistribution of ^{131}I-hMN-14 diabody 48 hours following injection. Samples were taken from tumor and normal tissues, including liver, spleen, kidney, lung, blood, stomach, small intestine and large intestine. The amount of ^{131}I-hMN-14 diabody is displayed as the percentage of the injected dose per gram of tissue (%ID/g).

[0019] *Figure 6* is a schematic representation of the hMN-14-0 polypeptide synthesized in *E. coli* from the hMN-14-0 expression plasmid, and the formation of a hMN-14 triabody. The nucleic acid construct encoding the unprocessed polypeptide contains sequences encoding the pelB signal peptide, the hMN-14 V_H and hMN-14V_K coding sequences, and a carboxyl terminal histidine affinity tag. The figure also shows a stick figure drawing of the mature polypeptide following proteolytic removal of the pelB signal peptide, and a stick figure drawing of a hMN-14 triabody, including CEA binding sites.

[0020] *Figure 7* shows the results of size-exclusion HPLC analysis of the hMN-14 triabody purification. The HPLC elution peak of hMN-14 triabody is at 9.01 minutes. Soluble proteins were purified by Ni-NTA IMAC followed by Q-Sepharose anion exchange chromatography. The flow-through fraction of the Q-Sepharose column was used for HPLC analysis. The retention times of hMN-14 diabody and hMN-14 F(ab')$_2$ are indicated with arrows.

[0021] *Figure 8* collectively shows a comparison of tumor uptake and blood clearance of hMN-14 diabody (Figure 8A), hMN-14 triabody (Figure 8B) and hMN-14 tetrabody (Figure 8C) over the first 96 hours following injection. The amount of ^{125}I-labeled proteins, measured as the percentage of the injected dose per gram of tissue (%ID/g), is plotted against time.

[0022] *Figure 9* is a schematic representation of the hMN-14-1G polypeptide synthesized in *E. coli* from the hMN-14-1G expression plasmid, and the formation of a hMN-14 tetrabody. The nucleic acid construct encoding the unprocessed polypeptide

contains sequences encoding the pelB signal peptide, the hMN-14 V_H and V_K coding sequences coupled by a single glycine residue, and the carboxyl terminal histidine affinity tag. The figure also shows a stick figure drawing of the mature polypeptide following proteolytic removal of the pelB signal peptide, and a stick figure drawing of a hMN-14 tetrabody, including CEA binding sites.

[0023] *Figure 10* shows the results of size-exclusion HPLC analysis of the hMN-14-1G polypeptide purification. Soluble proteins were purified by Ni-NTA IMAC followed by Q-Sepharose anion exchange chromatography. The flow-through fraction of the Q-Sepharose column was used for HPLC analysis. The HPLC elution peaks of diabody, triabody and tetrabody are indicated with arrows.

[0024] *Figure 11* is the nucleic acid sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of hMN-14-scFv-L5. Nucleic acid bases 1-66 encode the pelB signal peptide; 70-423 encode hMN-14 V_H; 424-438 encode the linker peptide (GGGGS); 439-759 encode hMN-14 V_K; and 766-783 encode the histidine affinity tag.

[0025] *Figure 12* is the deduced amino acid sequence of hMN-14 V_H (SEQ ID NO:3) and of hMN-14 V_K (SEQ ID NO:4).

[0026] *Figure 13* is the nucleic acid sequence (SEQ ID NO:5) and the deduced amino acid sequence (SEQ ID NO:6) of hMN-14-0. Nucleic acid bases 1-66 encode the pelB signal peptide; 70-423 encode hMN-14V_H; 424-744 encode hMN-14V_K; and 751-768 encode the histidine affinity tag.

[0027] *Figure 14* is the nucleic acid sequence (SEQ ID NO:7) and the deduced amino acid sequence (SEQ ID NO:8) of hMN-14-1G. Nucleic acid bases 1-66 encode the pelB signal peptide; 70-423 encode hMN-14V_H; 424-427 encode the linker peptide (G); 427-747 encode hMN-14V_K; and 754-771 encode the histidine affinity tag.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0028] Unless otherwise specified, "a" or "an" means "one or more".

[0029] One embodiment of this invention relates to multivalent, monospecific binding proteins. These binding proteins comprise two or more binding sites where each binding site has affinity for the same single target antigen. Each binding site is formed by the association of two or more single chain Fv (scFv) fragments. Each scFv comprises at least two variable domains derived from a humanized or human monoclonal antibody. The present invention further relates to monospecific diabodies, triabodies, and tetrabodies, which may further comprise a diagnostic or therapeutic agent, or a combination of two or more thereof.

[0030] Accordingly, the present invention provides a multivalent, monospecific binding protein comprising two or more binding sites having affinity for the same single target antigen, wherein said binding sites are formed by the association of two or more single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody. In certain embodiments, said monoclonal antibody is specific for a tumor-associated antigen.

[0031] Structurally, whole antibodies are composed of one or more copies of an Y-shaped unit that contains four polypeptide chains. Two chains are identical copies of a polypeptide, referred to as the heavy chain, and two chains are identical copies of a polypeptide, referred to as the light chain. Each polypeptide is encoded by individual DNA or by connected DNA sequences. The two heavy chains are linked together by one or more disulfide bonds and each light chain is linked to one of the heavy chains by one disulfide bond. Each chain has an N-terminal variable domain, referred to as V_H and V_L for the heavy and the light chains, respectively, and the non-covalent association of a pair of V_H and V_L, referred to as the Fv fragment, forms one antigen-binding site.

[0032] Discrete Fv fragments are prone to dissociation at low protein concentrations and under physiological conditions (Glockshuber *et al.*, *Biochemistry* **29**:1362-1367

(1990)), and therefore have limited use. To improve stability and enhance potential utility, recombinant single-chain Fv (scFv) fragments have been produced and studied extensively, in which the C-terminal of the V_H domain (or V_L) is joined to the N-terminal of the V_L domain (or V_H) via a peptide linker of variable length. (For a recent review, see Hudson and Kortt, *J. Immunol. Meth.* **231**:177-189 (1999)).

[0033] ScFvs with linkers greater than 12 amino acid residues in length (for example, 15 or 18 residue linkers) allow interactions between the V_H and V_L regions of the same polypeptide chain and generally form a mixture of monomers, dimers (termed diabodies) and small amounts of higher mass multimers (Kortt *et al.*, *Eur. J. Biochem.* **221**:151-157 (1994)). ScFvs with linkers of 5 or less amino acid residues, however, prohibit intramolecular association of the V_H and V_L regions of the same polypeptide chain, forcing pairing with V_H and V_L domains on a different polypeptide chain. Linkers between 3 and 12 amino acid residues form predominantly dimers (Atwell *et al.*, *Prot. Eng.* **12**:597-604 (1999)). ScFvs with linkers between 0 and 2 amino acid residues form trimeric (termed triabodies), tetrameric (termed tetrabodies) or higher oligomeric structures; however, the exact patterns of oligomerization appear to depend on the composition as well as the orientation of the V-domains, in addition to the linker length. For example, scFvs of the anti-neuraminidase antibody NC10 form predominantly trimers (V_H to V_L orientation) or tetramers (V_L to V_H orientation) with 0 amino acid residue linkers (Dolezal *et al.*, *Prot. Eng.* **13**:565-574 (2000)). ScFvs constructed from NC10 with 1 and 2 amino acid residue linkers, in the V_H to V_L orientation, form predominantly diabodies (Atwell *et al.*, *supra*); in contrast, the V_L to V_H orientation forms a mixture of tetramers, trimers, dimers, and higher mass multimers (Dolezal *et al.*, *supra*). ScFvs constructed from the anti-CD19 antibody HD37, in the V_H to V_L orientation, with a 0 amino acid residue linker form exclusively trimers, while the same construct with a 1 amino acid residue linker forms exclusively tetramers (Le Gall *et al.*, *FEBS Lett.* **453**:164-168 (1999)).

[0034] The non-covalent association of two or more scFv molecules can form functional diabodies, triabodies and tetrabodies, which are multivalent but monospecific. Monospecific diabodies are homodimers of the same scFv, where each scFv comprises the V_H domain from the selected antibody connected by a short linker to the V_L domain of the same antibody. A diabody is a bivalent dimer formed by the non-covalent association of two scFvs, yielding two Fv binding sites. A triabody results from the formation of a trivalent trimer of three scFvs, yielding three binding sites, and a tetrabody is a tetravalent tetramer of four scFvs, resulting in four binding sites. Several monospecific diabodies have been made using an expression vector that contains a recombinant gene construct comprising V_{H1}-linker-V_{L1}. (See Holliger *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:6444-6448 (1993); Atwell *et al.*, *Mol. Immunol.* **33**:1301-1312 (1996); Holliger *et al.*, *Nature Biotechnol.* **15**:632-636 (1997); Helfrich *et al.*, *Int. J. Cancer* **76**:232-239 (1998); Kipriyanov *et al.*, *Int. J. Cancer* 77:763-772 (1998); Holliger *et al.*, *Cancer Res.* **59**:2909-2916 (1999)). Methods of constructing scFvs are disclosed in U.S. Patent Nos. 4,946,778 and 5,132,405. Methods of producing multivalent, monospecific binding proteins based on scFv are disclosed in U.S. Patent Nos. 5,837,242 and 5,844,094, and PCT Application WO98/44001.

[0035] A humanized antibody is a recombinant protein in which the CDRs from an antibody from one species; e.g., a rodent antibody, is transferred from the heavy and light variable chains of the rodent antibody into human heavy and light variable domains. The constant domains of the antibody molecule is derived from those of a human antibody.

[0036] One embodiment of the present invention utilizes one monoclonal antibody, hMN-14, to produce antigen specific diabodies, triabodies, and tetrabodies. hMN-14 is a humanized monoclonal antibody (MAb) that binds specifically to CEA (Shevitz *et al.*, *J. Nucl. Med.* **S34**:217 (1993); and U.S. Patent No. 6,254,868). While the original MAbs were murine, humanized antibody reagents are now utilized to reduce the human anti-mouse antibody response. The variable regions of this antibody were engineered

into an expression construct (hMN-14-scFv-L5) as described in Example 1. As depicted in Figure 1, the nucleic acid construct (hMN-14-scFv-L5) for expressing an hMN-14 diabody encodes a polypeptide that possesses the following features:

(i) carboxyl terminal end of V_H linked to amino terminal end of V_K by the peptide linker Gly-Gly-Gly-Gly-Ser (G_4S) (the use of the G_4S peptide linker enables the secreted polypeptide to dimerize into a diabody, forming two binding sites for CEA);

(ii) pelB signal peptide sequence precedes the V_H gene to facilitate the synthesis of the polypeptide in the periplasmic space of *E. coli*; and

(iii) six histidine (6His) amino acid residues added to the carboxyl terminus to allow purification by IMAC.

The coding sequence of the nucleic acid (SEQ ID NO:1) and the corresponding deduced amino acid sequence (SEQ ID NO:2) of hMN-14-scFv-L5 are presented in Figure 11. Figure 1 also shows a stick figure drawing of the mature polypeptide following proteolytic removal of the pelB signal peptide, and a stick figure drawing of a hMN-14 diabody, including CEA binding sites.

[0037] A human antibody is an antibody obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.*, *Nature Genet.* **7**:13 (1994), Lonberg *et al.*, *Nature* **368**:856 (1994), and Taylor *et al.*, *Int. Immun.* **6**:579 (1994).

[0038] A fully human antibody also can be constructed by genetic or chromosomal transfection methods, as well as phage display technology, all of which are known in

the art. See for example, McCafferty *et al.*, *Nature* 348:552-553 (1990) for the production of human antibodies and fragments thereof in vitro, from immunoglobulin variable domain gene repertoires from unimmunized donors. In this technique, antibody variable domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. In this way, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats, for their review, see e.g. Johnson and Chiswell, *Curr. Opin. Struct. Biol.* 3:5564-571 (1993).

[0039] Human antibodies may also be generated by in vitro activated B cells. See U.S. Patent Nos. 5,567,610 and 5,229,275, which are hereby incorporated by reference herein in their entirety.

[0040] Accordingly, the present invention provides a multivalent, monospecific binding protein comprising two binding sites having affinity for the same single target antigen (termed a monospecific diabody), wherein said binding sites are formed by the association of two single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody. In certain embodiments, said monoclonal antibody is specific for a tumor-associated antigen. Preferably, said tumor-associated antigen is carcinoembryonic antigen (CEA).

[0041] In further embodiments, the humanized monoclonal antibody of this monospecific diabody is hMN-14. In such an embodiment, each scFv preferably comprises the V_H and the V_K regions of hMN-14. Optionally, each scFv further comprises an amino acid linker connecting the V_H and the V_K regions of hMN-14. In a preferred embodiment, each scFv comprises the amino acid sequence of SEQ ID NO:2.

[0042] Expression vectors were constructed through a series of sub-cloning procedures outlined in Figure 1 and described in Example 2. The expression cassette for monospecific hMN-14 binding proteins is shown schematically in Figure 1. The expression cassette may be contained in a plasmid, which is a small, double-stranded DNA forming an extra-chromosomal self-replicating genetic element in a host cell. A cloning vector is a DNA molecule that can replicate on its own in a microbial host cell. This invention describes vectors that expresses monospecific diabodies, triabodies, and tetrabodies. A host cell accepts a vector for reproduction and the vector replicates each time the host cell divides.

[0043] Accordingly, the present invention also provides an expression vector comprising a nucleotide sequence encoding a monospecific diabody as described.

[0044] A commonly used host cell is *Escherichia coli* (*E. coli*), however, other host cells are well known in the art, such as, for example, various bacteria, mammalian cells, yeast cells, and plant cells. In yeast, a number of vectors known to those of skill in the art can be used to introduce and express constructs in *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe* (fission yeast), *Pichia pastoris*, and *Hansenula polymorpha* (methylotropic yeasts) . In addition, a variety of mammalian expression vectors are commercially available. Further, a number of viral-based expression systems, such as adenovirus and retroviruses, can be utilized. By using such an expression system, large quantities of recombinant antibody can be produced using methods of the present invention, enabling their use as a viable delivery system.

[0045] Accordingly, the present invention also provides a host cell comprising an expression vector encoding a monospecific diabody as described.

[0046] When the cassette as shown in Figure 1 is expressed in *E. coli*, some of the polypeptides fold and spontaneously form soluble monospecific diabodies. The monospecific diabody shown in Figure 1 has two polypeptide chains that interact with each other to form two CEA binding sites having affinity for CEA antigens. Antigens

are bound by specific antibodies to form antigen-antibody complexes, which are held together by the non-covalent interactions of antigen and antibody molecules.

[0047] In this embodiment, two polypeptides comprising the V_H region of the hMN-14 MAb connected to the V_K region of the hMN-14 MAb by a five amino acid residue linker are utilized. Each polypeptide forms one half of the hMN-14 diabody. The coding sequence of the nucleic acid (SEQ ID NO:1) and the corresponding deduced amino acid sequence (SEQ ID NO:2) of each polypeptide are presented in Figure 11.

[0048] In the case of triabodies, when the cassette as shown in Figure 6 is expressed in *E. coli*, some of the polypeptides spontaneously form soluble monospecific triabodies. The monospecific triabody shown in Figure 6 has three polypeptide chains that interact with each other to form three CEA binding sites having high affinity for CEA antigens. Each of the three polypeptides comprise the V_H region of the hMN-14 MAb connected to the V_K region of the hMN-14 MAb, without a linker. Each polypeptide forms one third of the hMN-14 triabody. The coding sequence of the nucleic acid (SEQ ID NO:5) and the corresponding deduced amino acid sequence (SEQ ID NO:6) of each polypeptide is presented in Figure 13.

[0049] Accordingly, the present invention provides a multivalent, monospecific binding protein comprising three binding sites having affinity for the same single target antigen (termed a monospecific triabody), wherein said binding sites are formed by the association of three single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody. In certain embodiments, said monoclonal antibody is specific for a tumor-associated antigen. Preferably, said tumor-associated antigen is carcinoembryonic antigen (CEA).

[0050] In further embodiments, the humanized monoclonal antibody of this monospecific triabody is hMN-14. In such an embodiment, each scFv preferably comprises the V_H and the V_K regions of hMN-14. In certain embodiments, each scFv

comprises the amino acid sequence of SEQ ID NO:6. The present invention also provides an expression vector comprising a nucleotide sequence encoding the monospecific triabody and a host cell comprising this expression vector.

[0051] In the case of tetrabodies, when the cassette as shown in Figure 9 is expressed in *E. coli*, some of the polypeptides spontaneously form soluble monospecific tetrabodies. The monospecific tetrabody shown in Figure 9 has four polypeptide chains that interact with each other to form four CEA binding sites having high affinity for CEA antigens. Each of the four polypeptides comprise the V_H polypeptide of the hMN-14 MAb connected to the V_K polypeptide of the hMN-14 MAb by a single amino acid residue linker. Each polypeptide forms one fourth of the hMN-14 tetrabody. The coding sequence of the nucleic acid (SEQ ID NO:7) and the corresponding deduced amino acid sequence (SEQ ID NO:8) of each polypeptide is contained in Figure 14.

[0052] Accordingly, the present invention provides a multivalent, monospecific binding protein comprising four binding sites having affinity for the same single target antigen (termed a monospecific tetrabody), wherein said binding sites are formed by the association of four single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody. In certain embodiments, said monoclonal antibody is specific for a tumor-associated antigen. Preferably, said tumor-associated antigen is carcinoembryonic antigen (CEA).

[0053] In further embodiments, the humanized monoclonal antibody is hMN-14. In such an embodiment, each scFv preferably comprises the V_H and the V_K regions of hMN-14. Optionally, each scFv further comprises an amino acid linker connecting the V_H and the V_K regions of hMN-14. In certain embodiments, each scFv comprises the amino acid sequence of SEQ ID NO:8. The present invention also provides an expression vector comprising a nucleotide sequence encoding the monospecific tetrabody and a host cell comprising this expression vector.

[0054] In a preferred embodiment, the monospecific diabodies, triabodies, and tetrabodies of the present invention are used for direct targeting of diagnostic or therapeutic agents to CEA positive tumors. Other tumor-associated antigens may also be targeted, such as A3, A33, BrE3, CD1, CD1a, CD3, CD5, CD15, CD19, CD20, CD21, CD22, CD23, CD30, CD45, CD74, CD79a, CEA, CSAp, EGFR, EGP-1, EGP-2, Ep-CAM, Ba 733, HER2/neu, KC4, KS-1, KS1-4, Le-Y, MAGE, MUC1, MUC2, MUC3, MUC4, PAM-4, PSA, PSMA, RS5, S100, TAG-72, tenascin, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, VEGF, 17-1A, an angiogenesis marker, a cytokine, an immunomodulator, an oncogene marker and an oncogene product. The monospecific molecules bind selectively to targeted antigens and as the number of binding sites on the molecule increases, the affinity for the target cell increases. A stronger affinity allows the compositions of the present invention to remain at the desired location containing the target antigen for a longer time. Moreover, free unbound antibody molecules are cleared from the body quickly, thereby minimizing exposure of normal tissues to potentially harmful agents.

[0055] Tumor-associated markers have been categorized by Herberman (see, *e.g.*, Immunodiagnosis of Cancer, in THE CLINICAL BIOCHEMISTRY OF CANCER, Fleisher ed., American Association of Clinical Chemists, 1979) in a number of categories including oncofetal antigens, placental antigens, oncogenic or tumor virus associated antigens, tissue associated antigens, organ associated antigens, ectopic hormones and normal antigens or variants thereof. Occasionally, a sub-unit of a tumor-associated marker is advantageously used to raise antibodies having higher tumor-specificity, *e.g.*, the beta-subunit of human chorionic gonadotropin (HCG) or the gamma region of carcinoembryonic antigen (CEA), which stimulate the production of antibodies having a greatly reduced cross-reactivity to non-tumor substances as disclosed in U.S. Patent Nos. 4,361,644 and 4,444,744. Markers of tumor vasculature (*e.g.*, VEGF), of tumor necrosis, of membrane receptors (*e.g.*, folate receptor, EGFR), of transmembrane antigens (*e.g.*, PSMA), and of oncogene products can also serve as suitable tumor-associated targets for antibodies or antibody fragments. Markers of normal cell

constituents which are overexpressed on tumor cells, such as B-cell complex antigens, as well as cytokines expressed by certain tumor cells (*e.g.*, IL-2 receptor in T-cell malignancies) are also suitable targets for the antibodies and antibody fragments of this invention.

[0056] The BrE3 antibody is described in Couto *et al.*, *Cancer Res.* **55**:5973s-5977s (1995). The EGP-1 antibody is described in U.S. Provisional Application No. 60/360,229, some of the EGP-2 antibodies are cited in Staib *et al.*, *Int. J. Cancer* **92**:79-87 (2001) ; and Schwartzberg *et al.*, *Crit. Rev. Oncol. Hematol.* **40**:17-24 (2001). The KS -1 antibody is cited in Koda *et al.*, *Anticancer Res.* **21**:621-627 (2001); the A33 antibody is cited in Ritter *et al.*, *Cancer Res.* **61**:6854-6859 (2001); Le(y) antibody B3 is described in Di Carlo *et al.*, *Oncol. Rep.* **8**:387-392 (2001); and the A3 antibody is described in Tordsson *et al.*, *Int. J. Cancer* **87**:559-568 (2000).

[0057] Also of use are antibodies against markers or products of oncogenes, or antibodies against angiogenesis factors, such as VEGF. VEGF antibodies are described in U.S. Patent Nos. 6,342,221, 5,965,132 and 6,004,554, and are incorporated by reference in their entirety. Antibodies against certain immune response modulators, such as antibodies to CD40, are described in Todryk *et al.*, *J. Immunol. Meth.* **248**:139-147 (2001) and Turner *et al.*, *J. Immunol.* **166**:89-94 (2001). Other antibodies suitable for combination therapy include anti-necrosis antibodies as described in Epstein *et al.*, see *e.g.*, U.S. Patent Nos. 5,019,368; 5,882,626; and 6,017,514.

[0058] Accordingly, the present invention provides multivalent, monospecific binding proteins as described, comprising at least 2 variable domains derived from a humanized or human monoclonal antibody specific for a tumor-associated antigen associated with a disease state selected from the group consisting of a carcinoma, a melanoma, a sarcoma, a neuroblastoma, a leukemia, a glioma, a lymphoma and a myeloma. Said tumor-associated antigen may be associated with a type of cancer selected from the group consisting of acute lymphoblastic leukemia, acute myelogenous leukemia, biliary, breast, cervical, chronic lymphocytic leukemia, chronic myelogenous

leukemia, colorectal, endometrial, esophageal, gastric, head and neck, Hodgkin's lymphoma, lung, medullary thyroid, non-Hodgkin's lymphoma, ovarian, pancreatic, prostrate, and urinary bladder. Said tumor-associated antigen may be selected from the group consisting of A3, A33, BrE3, CD1, CD1a, CD3, CD5, CD15, CD19, CD20, CD21, CD22, CD23, CD30, CD45, CD74, CD79a, CEA, CSAp, EGFR, EGP-1, EGP-2, Ep-CAM, Ba 733, HER2/neu, KC4, KS-1, KS1-4, Le-Y, MAGE, MUC1, MUC2, MUC3, MUC4, PAM-4, PSA, PSMA, RS5, S100, TAG-72, tenascin, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, VEGF, 17-1A, an angiogenesis marker, a cytokine, an immunomodulator, an oncogene marker and an oncogene product. In a preferred embodiment, said tumor-associated antigen is carcinoembryonic antigen (CEA). In a preferred embodiment, the humanized monoclonal antibody is hMN-14.

[0059] A further embodiment of the invention involves using the inventive antibody or antibody fragment for detection, diagnosing and/or treating diseased tissues (*e.g.*, cancers), comprising administering an effective amount of a bivalent, trivalent, or tetravalent antibody or antibody fragment comprising at least two arms that specifically bind a targeted tissue.

[0060] Accordingly, the present invention provides multivalent, monospecific binding proteins as described, further comprising at least one agent selected from the group consisting of a diagnostic agent, a therapeutic agent, and combinations of two or more thereof. Said diagnostic agent may selected from the group consisting of a conjugate, a radionuclide, a metal, a contrast agent, a tracking agent, a detection agent, and combinations of two or more thereof.

[0061] In certain embodiments comprising a diagnostic radionuclide, said radionuclide is selected from the group consisting of ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{32}P, ^{51}Mn, ^{52}Fe, ^{52m}Mn, ^{55}Co, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, ^{86}Y, ^{89}Zr, ^{90}Y, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{110}In, ^{111}In, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}Gd$, ^{177}Lu, ^{186}Re, ^{188}Re, a gamma-emitter, a beta-emitter, a positron-emitter, and combinations of two or more

thereof. In certain other embodiments, said radionuclide is selected from the group consisting of ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{97}Ru, ^{99m}Tc, ^{111}In, ^{114m}In, ^{123}I, ^{125}I, ^{131}I, ^{169}Yb, ^{197}Hg, ^{201}Tl, and combinations of two or more thereof.

[0062] In certain embodiments comprising a metal, said metal is selected from the group consisting of gadolinium, iron, chromium, copper, cobalt, nickel, dysprosium, rhenium, europium, terbium, holmium, neodymium, and combinations of two or more thereof.

[0063] In certain embodiments comprising a contrast agent, said contrast agent may be a MRI contrast agent, a CT contrast agent, or an ultrasound contrast agent. A contrast agent may be selected from the group consisting of agadolinium ions, lanthanum ions, manganese ions, iron , chromium, copper, cobalt, nickel, dysporsium, rhenium, europium, terbium, holmium, neodymium, another comparable contrast agent, and combinations of two or more thereof.

[0064] In certain embodiments comprising a tracking agent, said tracking agent is selected from the group consisting of iodine compounds, barium compounds, gallium compounds, thallium compounds, barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamide, iodipamide, iodoxamic acid, iogulamide, iohexol, iopamidol, iopanoic acid, ioprocemic acid, iosefamic acid, ioseric acid, iosulamide meglumine, iosemetic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, thallous chloride, and combinations of two or more thereof.

[0065] In certain embodiments comprising a detection agent, said detection agent is selected from the group consisting of an enzyme, a fluorescent compound, a chemiluminescent compound, a bioluminescent compound, a radioisotope, and combinations of two or more thereof.

[0066] In certain embodiments comprising a therapeutic agent, said therapeutic agent is selected from the group consisting of a radionuclide, a chemotherapeutic drug,

a cytokine, a hormone, a growth factor, a toxin, an immunomodulator, and combinations of two or more thereof.

[0067] In certain embodiments comprising a therapeutic radionuclide is selected from the group consisting of ^{32}P, ^{33}P, ^{47}Sc, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{77}As, ^{89}Sr, ^{90}Y, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{111}Ag, ^{111}In, ^{125}I, ^{131}I, ^{142}Pr, ^{143}Pr, ^{149}Pm, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{169}Er, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{194}Ir, ^{198}Au, ^{199}Au, ^{211}At, ^{211}Pb, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra, ^{225}Ac, and combinations of two or more thereof. In certain other embodiments, said radionuclide is selected from the group consisting of ^{58}Co, ^{67}Ga, ^{80m}Br, ^{99m}Tc, ^{103m}Rh, ^{109}Pt, ^{111}In, ^{119}Sb, ^{125}I, ^{161}Ho, ^{189m}Os and ^{192}Ir, ^{152}Dy, ^{211}At, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{215}Po, ^{217}At, ^{219}Rn, ^{221}Fr, ^{223}Ra, ^{225}Ac, ^{255}Fm, and combinations of two or more thereof.

[0068] In certain embodiments comprising a chemotherapeutic drug, said chemotherapeutic drug is selected from the group consisting of vinca alkaloids, anthracyclines, epidophyllotoxins, taxanes, antimetabolites, alkylating agents, antibiotics, Cox-2 inhibitors, antimitotics, antiangiogenic agents, apoptotoic agents, doxorubicin, methotrexate, taxol, CPT-11, camptothecans, nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, pyrimidine analogs, purine analogs, platinum coordination complexes, hormones, and combinations of two or more thereof.

[0069] In certain embodiments comprising a toxin, said toxin is selected from the group consisting of ricin, abrin, ribonuclease, DNase I, *Staphylococcal* enterotoxin A, pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, *Pseudomonas* endotoxin, and combinations of two or more thereof.

[0070] In certain embodiments comprising an immunomodulator, said immunomodulator is selected from the group consisting of cytokines, stem cell growth factors, lymphotoxins, hematopoietic factors, colony stimulating factors, interferons, stem cell growth factors, erythropoietin, thrombopoietin, and combinations of two or more thereof.

[0071] A wide variety of diagnostic and therapeutic reagents can be advantageously conjugated to the antibodies of the invention. The therapeutic agents recited here are those agents that also are useful for administration separately with the multivalent binding proteins of the present invention as described herein. Therapeutic agents include, for example, chemotherapeutic drugs such as vinca alkaloids, anthracyclines, epidophyllotoxins, taxanes, antimetabolites, alkylating agents, antibiotics, Cox-2 inhibitors, antimitotics, antiangiogenic and apoptotoic agents, particularly doxorubicin, methotrexate, taxol, CPT-11, camptothecans, and others from these and other classes of anticancer agents, and the like. Other useful cancer chemotherapeutic drugs for the preparation of immunoconjugates and antibody fusion proteins include nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, COX-2 inhibitors, pyrimidine analogs, purine analogs, platinum coordination complexes, hormones, and the like. Useful therapeutic combinations may comprise other agents used to treat CEA-producing cancers, anti-HER2 antibodies (*e.g.*, Herceptin), and anti-EGF antibodies. Antibodies for combined use with the multivalent binding proteins of the present invention may be monoclonal, polyclonal, or humanized antibodies. Further suitable chemotherapeutic agents are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19th Ed. (Mack Publishing Co. 1995), and in GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed. (MacMillan Publishing Co. 1985), as well as revised editions of these publications. Other suitable therapeutic agents, include experimental drugs and drugs involved in clinical trials, as are known to those of skill in the art.

[0072] A toxin, such as *Pseudomonas* exotoxin, may also be complexed to or form the therapeutic agent portion of an immunoconjugate of the antibodies of the present invention. Other toxins suitably employed in the preparation of such conjugates or other fusion proteins, include ricin, abrin, ribonuclease (RNase), DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin (see, for example, Pastan *et al.*, *Cell* 47:641-648 (1986), and Goldenberg, *CA Cancer J. Clin.* 44:43964 (1994)).

Additional toxins suitable for use in the present invention are known to those of skill in the art and are disclosed in U.S. Patent No. 6,077,499, which is incorporated in its entirety by reference.

[0073] The diagnostic and therapeutic agents can include drugs, toxins, cytokines, conjugates with cytokines, hormones, growth factors, conjugates, radionuclides, contrast agents, metals, cytotoxic drugs, and immune modulators. For example, gadolinium metal is used for magnetic resonance imaging and fluorochromes can be conjugated for photodynamic therapy. Moreover, contrast agents can be MRI contrast agents, such as gadolinium ions, lanthanum ions, manganese ions, iron, chromium, copper, cobalt, nickel, dysprosium, rhenium, europium, terbium, holmium, neodymium or other comparable label, CT contrast agents, and ultrasound contrast agents.

[0074] In the methods of the invention, the targetable construct may comprise one or more radioactive isotopes useful for detecting diseased tissue. Particularly useful diagnostic radionuclides include, but are not limited to, ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{32}P, ^{51}Mn, ^{52}Fe, ^{52m}Mn, ^{55}Co, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, ^{86}Y, ^{89}Zr, ^{90}Y, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{110}In, ^{111}In, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}Gd$, ^{177}Lu, ^{186}Re, ^{188}Re, or other gamma-, beta-, or positron-emitters, preferably with a decay energy in the range of 20 to 4,000 keV, more preferably in the range of 25 to 4,000 keV, and even more preferably in the range of 20 to 1,000 keV, and still more preferably in the range of 70 to 700 keV. Total decay energies of useful positron-emitting radionuclides are preferably $<$ 2,000 keV, more preferably under 1,000 keV, and most preferably $<$ 700 keV.

[0075] Radionuclides useful as diagnostic agents utilizing gamma-ray detection include, but are not limited to: ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{97}Ru, ^{99m}Tc, ^{111}In, ^{114m}In, ^{123}I, ^{125}I, ^{131}I, ^{169}Yb, ^{197}Hg, and ^{201}Tl. Decay energies of useful gamma-ray emitting radionuclides are preferably 20-2000 keV, more preferably 60-600 keV, and most preferably 100-300 keV.

[0076] In the methods of the invention, the targetable construct may comprise one or more radioactive isotopes useful for treating diseased tissue. Particularly useful therapeutic radionuclides include, but are not limited to, ^{32}P, ^{33}P, ^{47}Sc, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{77}As, ^{89}Sr, ^{90}Y, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{111}Ag, ^{111}In, ^{125}I, ^{131}I, ^{142}Pr, ^{143}Pr, ^{149}Pm, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{169}Er, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{194}Ir, ^{198}Au, ^{199}Au, ^{211}At, ^{211}Pb, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra and ^{225}Ac. The therapeutic radionuclide preferably has a decay energy in the range of 20 to 6,000 keV, preferably in the ranges 60 to 200 keV for an Auger emitter, 100-2,500 keV for a beta emitter, and 4,000-6,000 keV for an alpha emitter.

[0077] Also preferred are radionuclides that substantially decay with Auger-emitting particles. Such radionuclides include, but are not limited, ^{58}Co, ^{67}Ga, ^{80m}Br, ^{99m}Tc, ^{103m}Rh, ^{109}Pt, ^{111}In, ^{119}Sb, ^{125}I, ^{161}Ho, ^{189m}Os and ^{192}Ir. Also preferred are radionuclides that substantially decay with generation of alpha-particles. Such radionuclides include, but are not limited to, ^{152}Dy, ^{211}At, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{215}Po, ^{217}At, ^{219}Rn, ^{221}Fr, ^{223}Ra, ^{225}Ac and ^{255}Fm. Decay energies of useful alpha-particle-emitting radionuclides are preferably 2,000-9,000 keV, more preferably 3,000-8,000 keV, and most preferably 4,000-7,000 keV.

[0078] The present invention antibodies and fragments thereof may include additional tracking agents. Radiopaque and contrast materials are used for enhancing X-rays and computed tomography, and include iodine compounds, barium compounds, gallium compounds, thallium compounds, *etc.* Specific compounds include barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamide, iodipamide, iodoxamic acid, iogulamide, iohexol, iopamidol, iopanoic acid, ioprocemic acid, iosefamic acid, ioseric acid, iosulamide meglumine, iosemetic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, and thallous chloride.

[0079] The present invention antibodies and fragments thereof also can be labeled with a fluorescent compound. The presence of a fluorescently-labeled MAb is

determined by exposing the target antigen binding protein to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine. Fluorescently-labeled antigen binding proteins are particularly useful for flow cytometry analysis.

[0080] Alternatively, antibodies and fragments thereof can be detectably labeled by coupling the binding protein to a chemiluminescent compound. The presence of the chemiluminescent-tagged MAb is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

[0081] Similarly, a bioluminescent compound can be used to label antibodies and fragments thereof. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

[0082] Alternatively, antibodies and fragments thereof can be detectably labeled by linking the antibody to an enzyme. When the antibody-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label antibody include malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

[0083] An immunomodulator, such as a cytokine, may also be conjugated to, or form the therapeutic agent portion of the antibody immunoconjugate, or be administered unconjugated to the chimeric, humanized, or human antibodies or fragments thereof of the present invention. As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, such as tumor necrosis factor (TNF), and hematopoietic factors, such as interleukins (*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-10, IL-12 and IL-18), colony stimulating factors (*e.g.*, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (*e.g.*, interferons-α, -β and -γ), the stem cell growth factor designated "S1 factor," erythropoietin and thrombopoietin. Examples of suitable immunomodulator moieties include IL-2, IL-6, IL-10, IL-12, IL-18, interferon-γ, TNF-α, and the like. Alternatively, subjects can receive naked antibodies and a separately administered cytokine, which can be administered before, concurrently or after administration of the naked antibodies. The antibody may also be conjugated to the immunomodulator. The immunomodulator may also be conjugated to a hybrid antibody consisting of one or more antibodies binding to different antigens.

[0084] A therapeutic or diagnostic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation. As an alternative, such peptides can be attached to the antibody component using a heterobifunctional cross-linker, such as *N*-succinyl 3-(2-pyridyldithio)proprionate (SPDP) (Yu *et al.*, *Int. J. Cancer* **56**: 244-248 (1994)). General techniques for such conjugation are well-known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991); Upeslacis *et al.*, "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.* (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 60-84 (Cambridge University Press 1995). Alternatively, the therapeutic or diagnostic agent can be conjugated via a carbohydrate moiety in the Fc region of the

antibody. The carbohydrate group can be used to increase the loading of the same peptide that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different peptide.

[0085] These agents are designed to diagnose and/or treat disorders in mammals. Mammals can include humans, domestic animals, and pets, such as cats and dogs. The mammalian disorders can include cancers, such as carcinomas, melanomas, sarcomas, neuroblastomas, leukemias, gliomas and myelomas. Exemplary types of cancers include, but are not limited to, biliary, breast, cervical, colorectal, endometrial, esophageal, gastric, head and neck, lung, medullary thyroid, ovarian, pancreatic, prostrate and urinary bladder.

[0086] Accordingly, the present invention provides a method of diagnosing the presence of a tumor, said method comprising administering to a subject suspected of having a tumor a detectable amount of a multivalent, monospecific binding protein as described, comprising a diagnostic agent as described, and monitoring the subject to detect any binding of the binding protein to a tumor.

[0087] The present invention further provides a method of treating a tumor, said method comprising administering to a subject in need thereof an effective amount of a multivalent, monospecific binding protein as described, comprising a diagnostic agent as described.

[0088] The present invention further provides a method of diagnosing the presence of a tumor, said method comprising administering to a subject suspected of having a tumor a detectable amount of a multivalent, monospecific binding protein as described, in combination with a detectable moiety that is capable of binding to said binding protein, and monitoring the subject to detect any binding of the binding protein to a tumor.

[0089] The present invention further provides a method of treating a tumor, said method comprising administering to a subject in need thereof an effective amount of a

multivalent, monospecific binding protein as described, in combination with a therapeutic agent. In preferred embodiments, said therapeutic agent is selected from the group consisting of a chemotherapeutic drug, a toxin, external radiation, a brachytherapy radiation agent, a radiolabeled protein, an anticancer drug and an anticancer antibody.

[0090] The present invention further provides a method of delivering one or more diagnostic agent, one or more therapeutic agent, or a combination of two or more thereof to a tumor, said method comprising administering to a subject in need thereof a multivalent, monospecific binding protein as described, further comprising at least one agent selected from the group consisting of a diagnostic agent, a therapeutic agent, and combinations of two or more thereof.

[0091] Delivering a diagnostic or a therapeutic agent to a target for diagnosis or treatment in accordance with the invention includes providing the binding protein with a diagnostic or therapeutic agent and administering to a subject in need thereof with the binding protein. Diagnosis further requires the step of detecting the bound proteins with known techniques.

[0092] Administration of the binding protein with diagnostic or therapeutic agents of the present invention to a mammal may be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering the binding protein by injection, the administration may be by continuous infusion or by single or multiple boluses.

[0093] The binding protein with the diagnostic or therapeutic agent may be provided as a kit for human or mammalian therapeutic and diagnostic use in a pharmaceutically acceptable injection vehicle, preferably phosphate-buffered saline (PBS) at physiological pH and concentration. The preparation preferably will be sterile, especially if it is intended for use in humans. Optional components of such kits include stabilizers,

buffers, labeling reagents, radioisotopes, paramagnetic compounds, second antibody for enhanced clearance, and conventional syringes, columns, vials, and the like.

[0094] Accordingly, the present invention also provides a kit for therapeutic and/or diagnostic use, said kit comprising at least one multivalent, monospecific binding protein as described, further comprising at least one agent selected from the group consisting of a diagnostic agent, a therapeutic agent, and combinations of two or more thereof, and additional reagents, equipment, and instructions for use.

EXAMPLES

[0095] The examples below are illustrative of embodiments of the current invention and should not be used, in any way, to limit the scope of the claims.

Example 1 - Construction of plasmids for expression of hMN-14 diabody in *E. coli*

[0096] Standard recombinant DNA methods were used to obtain hMN-14-scFv-L5 as follows. The hMN-14 V_H and V_K sequences were amplified from a vector constructed for expressing hMN-14 Fab' (Leung *et al.*, *Cancer Res.* 55:5968s-5972s (1995)) using the polymerase chain reaction (PCR) with Pfu polymerase. The hMN-14V_H sequence was amplified using the oligonucleotide primers specified below:

hMN-14V_H-Left

5' - CGTACCATGGAGGTCCAACTGGTGGAGA - 3' (SEQ ID NO:9)

hMN-14V_H-Right (G_4S)

5'-CATAGGATCCACCGCCTCCGGAGACGGTGACCGGGGT - 3' (SEQ ID NO:10)

[0097] The left PCR primer contains a 5' NcoI restriction site. The right PCR primer contains a sequence for a 5 amino acid residue linker (G_4S) and a BamHI restriction site. The PCR product was digested with NcoI and BamHI and ligated, in frame with the pelB signal peptide sequence, into NcoI/BamHI digested pET-26b vector to generate hMN-14V_HL5-pET26. The hMN-14V_K sequence was amplified using the oligonucleotide primers specified below:

hMN-14V$_K$-Left

5' - CTGAGGATCCGACATCCAGCTGACCCAGAG - 3' (SEQ ID NO:11)

hMN-14V$_K$-Right

5' - GCTACTCGAGACGTTTGATTTCCACCTTGG - 3' (SEQ ID NO:12)

[0098] The left and right PCR primers contain BamHI and XhoI restriction sites, respectively. The PCR product was digested with XhoI and BamHI and ligated, in frame with the hMN-14V$_H$, G$_4$S linker and 6His sequences, into the XhoI/BamHI digested hMN-14V$_H$L5-pET26 construct to generate the expression construct hMN-14-scFv-L5. The DNA sequence of this construct was verified by automated DNA sequencing, and is listed in Figure 11. The nucleic acid construct, hMN-14-scFv-L5, is illustrated in Figure 1.

Example 2 - Expression of hMN-14 diabody in *E. coli*

[0099] The hMN-14-scFv-L5 construct was used to transform BL21(P-LysS) *E. coli*. Culture conditions, induction, and purification were carried as described below. Competent *E. coli* BL21(P-Lys-S) cells were transformed with hMN-14-scFv-L5 by standard methods. Cultures were shaken in 2xYT media supplemented with 100 μg/ml kanamycin sulphate and 34 μg/ml chloramphenicol and grown at 37°C to OD$_{600}$ of 1.6–1.8. An equal volume of room temperature 2xYT media supplemented with antibiotics and 0.8 M sucrose was added to the cultures, which were then transferred to 20°C. After 30 minutes at 20°C, expression was induced by the addition of 40 μM IPTG and continued at 20°C for 15-18 hours.

[0100] The expression of hMN-14 diabody was examined in (1) cell culture conditioned media; (2) soluble proteins extracted under non-denaturing conditions from the cell pellet following centrifugation; and (3) insoluble material remained in the pellet following several cycles of extraction and centrifugation.

[0101] Soluble proteins were extracted from bacterial cell pellets as follows. Pellets were frozen and thawed, then re-suspended in lysis buffer (2% Triton X-100; 300 mM

NaCl; 10 mM imidazole; 5 mM $MgSO_4$; 25 units/ml benzonase; 50 mM NaH_2PO_4 (pH 8.0)) using a volume equal to 1% of the culture volume. The suspension was homogenized by sonication, clarified by centrifugation, and loaded onto Ni-NTA IMAC columns. After being washed with buffer containing 20 mM imidazole, the columns were eluted with 100 mM imidazole buffer (100 mM imidazole; 50 mM NaCl; 25 mM Tris (pH 7.5)) and the eluate was further purified by affinity chromatography via binding to an anti-id antibody immobilized on Affi-gel.

[0102] The insoluble pelleted material was solubilized in denaturing Ni-NTA binding buffer (8 M urea; 10 mM imidazole; 0.1 M NaH_2PO_4; 10 mM Tris (pH 8.0)) and mixed with 1 ml of Ni-NTA agarose (Qiagen, Inc.). The mixture was rocked at room temperature for 1 hour, then the resin was washed once with 50 ml of the same buffer and loaded onto a column. The column was washed with 20 ml of the same buffer followed by 20 ml of wash buffer (8 M urea; 20 mM imidazole; 0.1 M NaH_2PO_4; 10 mM Tris (pH 8.0)). Bound proteins were eluted with 5 ml of denaturing elution buffer (8 M urea; 250 mM imidazole; 0.1 M NaH_2PO_4; 10 mM Tris (pH 8.0)).

[0103] Soluble proteins that bound to and were eluted from Ni-NTA resin were loaded on a WI2 anti-idiotype affinity column. The column was washed with PBS and the bound polypeptides were eluted with 0.1 M glycine; 0.1 M NaCl (pH 2.5) and neutralized immediately.

[0104] Although most of the hMN-14scFv expressed was present as insoluble protein, approximately 1.5 mg/L culture of soluble hMN-14scFv was purified from the soluble fraction. As shown by size-exclusion high performance liquid chromatography (HPLC), a predominant peak was observed (see Figures 2A and 2B) at 9.8 minutes for the IMAC purified as well as the affinity purified material. The retention time of hMN-14 Fab', which has a molecular weight of approximately 50 kDa, was 9.75 minutes as indicated on the x-axis of Figure 2B. The very similar retention time of hMN-14scFv indicates that it exists in solution as a dimer or diabody since the calculated molecular weight of the monomeric hMN-14scFv is 26 kDa. SDS-PAGE gel analysis (see Figure

3A) shows a single band of the predicted size at 26 kDa, and the isoelectric focusing (IEF) gel analysis (see Figure 3B) yields a band with pI of 8.2, close to the calculated pI of 7.9. A competitive ELISA showed that the hMN-14 diabody is functional and displays excellent binding properties.

[0105] Nude mice bearing the CEA positive GW-39 tumor were injected with ^{131}I-labeled hMN-14 diabody and the biodistribution was analyzed at various times following injection. While a significant amount of the diabody remained associated with the tumor for more than 96 hours, much of the free diabody cleared the blood rapidly as illustrated in Figure 4. Figure 5 shows the percentage of the injected dose that is associated with the tumor and with normal tissues, such as liver, spleen, kidney, lungs, blood, stomach, small intestine, and large intestine, at 48 hours after the injection. The amount of the injected dose in each normal tissue is very low when compared to the amount in the tumor. Table 1 summarizes the relative amounts of activity increased in the tumor over the listed normal tissues at 24, 48 and 72 hours (*e.g.*, at 24 hours, the tumor has 22.47 times as much radioactivity as does the liver).

Table 1. Tumor to non-tumor ratios

	24 hours	48 hours	72 hours
Tumor	1.00	1.00	1.00
Liver	22.47	31.85	28.32
Spleen	25.41	39.51	41.03
Kidney	9.12	12.12	10.54
Lung	15.49	25.70	31.75
Blood	9.84	17.32	21.80
Stomach	9.98	17.50	23.13
Sm. Int.	37.23	65.60	50.58
Lg. Int.	35.87	66.54	45.66

Example 3 - Construction of a plasmid for the expression of hMN-14 triabody

[0106] An hMN-14scFv plasmid construct, hMN-14-0, was designed, produced and tested. The *E. coli* expression plasmid directs the synthesis of a single polypeptide possessing the following features: (1) the carboxyl terminal end of hMN-14V_H is directly linked to the amino terminal end of hMN-14V_K without any additional amino acids (the use of the zero linker enables the secreted polypeptide to form a trimeric structure called a triabody, forming three binding sites for CEA); (2) a pelB signal peptide sequence precedes the V_H gene to facilitate the synthesis of the polypeptide in the periplasmic space of *E. coli*; and (3) six histidine (6His) residues are added to the carboxyl terminus to allow purification by IMAC. A schematic representation of the polypeptide and triabody are shown in Figure 6.

[0107] Standard recombinant DNA methods were used to obtain the hMN-14-0 construct. The hMN-14 V_H and V_K sequences were amplified from the hMN-14scFv-L5 construct, using PCR with Pfu polymerase. The hMN-14V_H sequence was amplified using the oligonucleotide primers specified below:

hMN-14V_H-Left

5' - CGTACCATGGAGGTCCAACTGGTGGAGA - 3' (SEQ ID NO:13)

hMN-14V_H-0 Right

5' - GATATCGGAGACGGTGACCGGG - 3' (SEQ ID NO:14)

[0108] The left PCR primer, which was previously used for the construction of hMN-14scFv-L5, contains a 5' NcoI restriction site. The right PCR primer contains EcoRV restriction site. The PCR product was cloned into PCR cloning vector pGemT (Promega).

[0109] The hMN-14V_K sequence was amplified using the oligonucleotide primers specified below:

hMN-14V_K-0 Left

5' - GATATCCAGCTGACCCAGAGCC - 3' (SEQ ID NO:15)

hMN-14V_K-Right

5' - GCTACTCGAGACGTTTGATTTCCACCTTGG - 3' (SEQ ID NO:16)

[0110] The left PCR primer contains an EcoRV restriction site. The right primer, which was previously used for the construction of hMN-14scFv-L5, contains an XhoI restriction site. The PCR product was cloned into pGemT vector. The V_K-0 sequence was excised from the V_K-0-pGemT construct with EcoRV and SalI and ligated into the same sites of the V_H-0-pGemT construct to generate hMN-14-0 in pGemT. The V_H-V_K sequence was excised with NcoI and XhoI and transferred to pET26b to generate the hMN-14 triabody expression construct hMN-14-0. The DNA sequence of this construct was verified by automated DNA sequencing, and is listed in Figure 13. The nucleic acid construct, hMN-14scFv-0, is illustrated in Figure 6.

Example 4 - Expression of hMN-14 triabody in *E. coli*

[0111] The hMN-14-0 construct was used to transform BL21(P-LysS) *E. coli*. Culture conditions, induction, and purification were carried out similar to those described for the hMN-14 diabody in Example 2, except that the hMN-14-0 triabody was purified by Q-Sepharose anion exchange chromatography, instead of affinity

chromatography. As expected, hMN-14-0 formed predominantly triabodies (~80 kDa).

[0112] Approximately 2.4 mg/L culture of soluble hMN-14 triabody was purified from the soluble cell fraction of induced cultures. As shown by size-exclusion HPLC (see Figure 7), a predominant peak was observed at 9.01 minutes for material purified by IMAC and mono-Q anion exchange chromatography. By comparison, the retention times of hMN-14 diabody (~52 kDa) and hMN-14 F(ab')$_2$ (~100 kDa) were 9.6 minutes and 8.44 minutes, respectively. The fact that the retention time of hMN-14-0 is exactly halfway between those of the 52 kDa and 100 kDa proteins indicates that it exists in solution as a trimer or triabody; since the calculated molecular weight of the monomeric hMN-14-0 polypeptide is ~26 kDa. Indeed, SDS-PAGE analysis shows a single band of the predicted 26 kDa.

[0113] Nude mice bearing the CEA positive GW-39 tumor were injected with ^{131}I-labeled hMN-14 triabody and the biodistribution was analyzed at various times following injection. Figure 8 shows hMN-14 triabody tumor uptake and retention are remarkably higher than that of hMN-14 diabody. After one hour, triabody accumulates in the tumor at approximately 60% of the level of the diabody. However, while the diabody decreases steadily after one hour, triabody tumor uptake increases to a maximal level achieved between 24 and 48 hours. The maximal triabody tumor uptake (24-48 hours) is more than twice that of the diabody (1 hour). The tumor retention is also significantly longer for the triabody compared to diabody as the triabody may exhibit trivalent tumor binding by utilizing all three CEA binding sites. An additional factor that likely has a significant influence on tumor uptake is molecular size. As depicted in Figure 8, blood clearance for the 80 kDa triabody is much slower than that of the 54 kDa diabody. This allows the triabody a much longer time to interact with the tumor, as compared to the diabody, and thus achieve higher levels of tumor uptake. The triabody's delayed blood clearance undoubtedly contributes to its superior tumor residence. However, other factors, including increased avidity due to multivalency or

improved *in vivo* stability, may also contribute. Tumor to non-tumor ratios increased with time for all tissues (Table 2). The ratios were substantial at the later time points.

Table 2. Tumor to non tumor ratios for hMN-14 triabody.

	24 hours	48 hours	72 hours
Liver	15.7	45.9	110.3
Spleen	13.7	39.9	96.9
Kidney	8.4	25.2	52.8
Lung	6.0	18.7	44.4
Blood	3.4	12.4	54.8
Stomach	11.3	15.0	62.4
Sm. Int.	28.3	78.5	204.7
Lg Int.	40.3	105.0	195.1

Example 5 - Construction of plasmids for the expression of hMN-14 Tetrabodies

[0114] An hMN-14scFv plasmid construct, hMN-14-1G, was designed, produced and tested. The *E. coli* expression plasmid directs the synthesis of a single polypeptide possessing the following features: (1) the carboxyl terminal end of hMN-14V_H is linked to the amino terminal end of hMN-14V_K by a single glycine residue (the use of the 1G linker enables some of the secreted polypeptide to form a tetrameric structure called a tetrabody, forming four binding sites for CEA); (2) a pelB signal peptide sequence precedes the V_H gene to facilitate the synthesis of the polypeptide in the periplasmic space of *E. coli*; and (3) six histidine (6His) residues are added to the carboxyl terminus to allow purification by IMAC. A schematic representation of the polypeptide and tetrabody are shown in Figure 9.

[0115] Standard recombinant DNA methods were used to obtain the hMN-14-1G construct. The hMN-14 V_H and V_K sequences were amplified from the hMN-14scFv-L5 construct, using PCR with Pfu polymerase. The hMN-14V_H sequence was amplified using the oligonucleotide primers specified below:

hMN-14V_H-Left

5' - CGTACCATGGAGGTCCAACTGGTGGAGA - 3' (SEQ ID NO:17)

hMN-14V$_H$-1G Right

5' - GCTGGATATCACCGGAGACGGTGACCGGGGTCC - 3' (SEQ ID NO:18)

[0116] The left PCR primer, which was previously used for the construction of hMN-14scFv-L5, contains a 5' NcoI restriction site. The right PCR primer contains the coding sequence for a single glycine and an EcoRV restriction site. The PCR product was cloned into the PCR cloning vector pGemT (Promega). The hMN-14V$_K$-0 sequence (see Example 3) was excised from the hMN-14V$_K$-0-pGemT construct with EcoRV and SalI and ligated into the same sites of the hMN-14V$_H$-1G-pGemT construct to generate hMN-14-1G in pGemT. The V$_H$-1G-V$_K$ sequence was excised with NcoI and XhoI and transferred to pET26b to generate the hMN-14 tetrabody expression construct hMN-14-1G. The DNA sequence of this construct was verified by automated DNA sequencing, and is listed in Figure 14. The nucleic acid construct, hMN-14scFv-1G, is illustrated in Figure 9.

Example 6 - Expression of hMN-14 Tetrabodies in *E. coli*

[0117] The hMN-14-1G construct was used to transform BL21(P-LysS) *E. coli*. Culture conditions, induction, and purification were carried out similar to those described for the hMN-14 diabody in Example 2, except that the hMN-14 tetrabody was purified by Q-Sepharose anion exchange chromatography, instead of affinity chromatography. Soluble expression levels were high, greater than 2 mg of soluble product was isolated per liter of culture. Size exclusion HPLC analysis (see Figure 10) demonstrated that the hMN-14-1G product exists as a mixture of diabody (53 kDa), triabody (80 kDa) and tetrabody (105-120 kDa). The tetrabody could be isolated in relatively pure form by gel filtration chromatography. However, after several days at 2-8°C, it gradually reverted to a mixture of diabody, triabody and tetrabody similar to that shown in Figure 10.

Example 7 –Tumor Uptake of hMN-14 Diabody, Triabody, and Tetrabody

[0118] Tumor targeting was evaluated in mice bearing CEA-positive human colon tumor xenografts using radioiodinated samples. At 24 h, the diabody (obtained from hMN-14-L5) showed 2.7% injected dose per gram (ID/g) in the tumor, 0.3% in the blood, and 0.1 to 0.4% in all other organs. For the triabody (obtained from hMN-14-0), the tumor uptake was 12.0, 12.2, 11.1, and 7.1% ID/g at 24, 48, 72 and 96 h, respectively, with tumor to blood ratios increasing from 3.4 at 24 h to 12.4 at 48 h, and up to 55 at 96 h. The tetrabody (obtained from hMN-14-1G) displayed the highest tumor uptake among the three, reaching 25.4% ID/g at 24 h with a tumor to blood ratio of 3.9 and decreasing to 17.1% at 72 h, with a tumor to blood ratio of 29.3. These biodistribution results are in agreement with the respective molecular size and multivalency of the three novel scFv-based agents, all of which, and in particular the hMN-14 triabody, are especially useful for imaging and therapeutic applications.

[0119] While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention. The present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The examples provided here are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[0120] The disclosure of all publications cited above are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually.

That which is claimed is:

1. A multivalent, monospecific binding protein comprising two or more binding sites having affinity for the same single target antigen, wherein said binding sites are formed by the association of two or more single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody.

2. The binding protein according to claim 1, wherein said monoclonal antibody is specific for a tumor-associated antigen.

3. The binding protein according to claim 2, wherein said tumor-associated antigen is associated with a disease state selected from the group consisting of a carcinoma, a melanoma, a sarcoma, a neuroblastoma, a leukemia, a glioma, a lymphoma and a myeloma.

4. The binding protein according to claim 2, wherein said tumor-associated antigen is associated with a type of cancer selected from the group consisting of acute lymphoblastic leukemia, acute myelogenous leukemia, biliary, breast, cervical, chronic lymphocytic leukemia, chronic myelogenous leukemia, colorectal, endometrial, esophageal, gastric, head and neck, Hodgkin's lymphoma, lung, medullary thyroid, non-Hodgkin's lymphoma, ovarian, pancreatic, prostrate, and urinary bladder.

5. The binding protein according to claim 2, wherein said tumor-associated antigen is selected from the group consisting of A3, A33, BrE3, CD1, CD1a, CD3, CD5, CD15, CD19, CD20, CD21, CD22, CD23, CD30, CD45, CD74, CD79a, CEA, CSAp, EGFR, EGP-1, EGP-2, Ep-CAM, Ba 733, HER2/neu, KC4, KS-1, KS1-4, Le-Y, MAGE, MUC1, MUC2, MUC3, MUC4, PAM-4, PSA, PSMA, RS5, S100, T101, TAG-72, tenascin, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis

antigens, VEGF, 17-1A, an angiogenesis marker, a cytokine, an immunomodulator, an oncogene marker and an oncogene product.

6. The binding protein according to claim 2, wherein said tumor-associated antigen is carcinoembryonic antigen (CEA).

7. The binding protein according to claim 6, wherein the humanized monoclonal antibody is hMN-14.

8. The binding protein of claim 1, further comprising at least one agent selected from the group consisting of a diagnostic agent, a therapeutic agent, and combinations of two or more thereof.

9. The binding protein of claim 8, wherein said diagnostic agent is selected from the group consisting of a conjugate, a radionuclide, a metal, a contrast agent, a tracking agent, a detection agent, and combinations of two or more thereof.

10. The binding protein of claim 9, wherein said radionuclide is selected from the group consisting of ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{32}P, ^{51}Mn, ^{52}Fe, ^{52m}Mn, ^{55}Co, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, ^{86}Y, ^{89}Zr, ^{90}Y, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{110}In, ^{111}In, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}Gd$, ^{177}Lu, ^{186}Re, ^{188}Re, a gamma-emitter, a beta-emitter, a positron-emitter, and combinations of two or more thereof.

11. The binding protein of claim 9, wherein said radionuclide is selected from the group consisting of ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{97}Ru, ^{99m}Tc, ^{111}In, ^{114m}In, ^{123}I, ^{125}I, ^{131}I, ^{169}Yb, ^{197}Hg, ^{201}Tl, and combinations of two or more thereof.

12. The binding protein of claim 9, wherein said metal is selected from the group consisting of gadolinium, iron, chromium, copper, cobalt, nickel, dysprosium,

rhenium, europium, terbium, holmium, neodymium, and combinations of two or more thereof.

13. The binding protein of claim 9, wherein said contrast agent is a MRI contrast agent.

14. The binding protein of claim 9, wherein said contrast agent is a CT contrast agent.

15. The binding protein of claim 9, wherein said contrast agent is an ultrasound contrast agent.

16. The binding protein of claim 9, wherein said contrast agent is selected from the group consisting of agadolinium ions, lanthanum ions, manganese ions, iron, chromium, copper, cobalt, nickel, dysporsium, rhenium, europium, terbium, holmium, neodymium, another comparable contrast agent, and combinations of two or more thereof.

17. The binding protein of claim 9, wherein said tracking agent is selected from the group consisting of iodine compounds, barium compounds, gallium compounds, thallium compounds, barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamide, iodipamide, iodoxamic acid, iogulamide, iohexol, iopamidol, iopanoic acid, ioprocemic acid, iosefamic acid, ioseric acid, iosulamide meglumine, iosemetic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, thallous chloride, and combinations of two or more thereof.

18. The binding protein of claim 9, wherein said detection agent is selected from the group consisting of an enzyme, a fluorescent compound, a chemiluminescent

compound, a bioluminescent compound, a radioisotope, and combinations of two or more thereof.

19. The binding protein of claim 8, wherein said therapeutic agent is selected from the group consisting of a radionuclide, a chemotherapeutic drug, a cytokine, a hormone, a growth factor, a toxin, an immunomodulator, and combinations of two or more thereof.

20. The binding protein of claim 19, wherein said radionuclide is selected from the group consisting of ^{32}P, ^{33}P, ^{47}Sc, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{75}Se, ^{77}As, ^{89}Sr, ^{90}Y, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{111}Ag, ^{111}In, ^{125}I, ^{131}I, ^{142}Pr, ^{143}Pr, ^{149}Pm, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{169}Er, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{194}Ir, ^{198}Au, ^{199}Au, ^{211}At, ^{211}Pb, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra, ^{225}Ac, and combinations of two or more thereof.

21. The binding protein of claim 19, wherein said radionuclide is selected from the group consisting of ^{58}Co, ^{67}Ga, ^{80m}Br, ^{99m}Tc, ^{103m}Rh, ^{109}Pt, ^{111}In, ^{119}Sb, ^{125}I, ^{161}Ho, ^{189m}Os and ^{192}Ir, ^{152}Dy, ^{211}At, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{215}Po, ^{217}At, ^{219}Rn, ^{221}Fr, ^{223}Ra, ^{225}Ac, ^{255}Fm, and combinations of two or more thereof.

22. The binding protein of claim 19, wherein said chemotherapeutic drug is selected from the group consisting of vinca alkaloids, anthracyclines, epidophyllotoxins, taxanes, antimetabolites, alkylating agents, antibiotics, Cox-2 inhibitors, antimitotics, antiangiogenic agents, apoptotoic agents, doxorubicin, methotrexate, taxol, CPT-11, camptothecans, nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, pyrimidine analogs, purine analogs, platinum coordination complexes, hormones, and combinations of two or more thereof.

23. The binding protein of claim 19, wherein said toxin is selected from the group consisting of ricin, abrin, ribonuclease, DNase I, *Staphylococcal* enterotoxin A,

pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, *Pseudomonas* endotoxin, and combinations of two or more thereof.

24. The binding protein of claim 19, wherein said immunomodulator is selected from the group consisting of cytokines, stem cell growth factors, lymphotoxins, hematopoietic factors, colony stimulating factors, interferons, stem cell growth factors, erythropoietin, thrombopoietin, and combinations of two or more thereof.

25. A multivalent, monospecific binding protein comprising two binding sites having affinity for the same single target antigen (termed a monospecific diabody), wherein said binding sites are formed by the association of two single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody.

26. The monospecific diabody according to claim 25, wherein said monoclonal antibody is specific for a tumor-associated antigen.

27. The monospecific diabody according to claim 26, wherein said tumor-associated antigen is carcinoembryonic antigen (CEA).

28. The monospecific diabody according to claim 25, wherein the humanized monoclonal antibody is hMN-14.

29. The monospecific diabody according to claim 28, wherein each scFv comprises the V_H and the V_K regions of hMN-14.

30. The monospecific diabody according to claim 29, wherein each scFv further comprises an amino acid linker connecting the V_H and the V_K regions of hMN-14.

31. The monospecific diabody according to claim 30, wherein each scFv comprises the amino acid sequence of SEQ ID NO:2.

32. An expression vector comprising a nucleotide sequence encoding the monospecific diabody of claim 25.

33. A host cell comprising the expression vector of claim 32.

34. A multivalent, monospecific binding protein comprising three binding sites having affinity for the same single target antigen (termed a monospecific triabody), wherein said binding sites are formed by the association of three single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody.

35. The monospecific triabody according to claim 34, wherein said monoclonal antibody is specific for a tumor-associated antigen.

36. The monospecific triabody according to claim 35, wherein said tumor-associated antigen is carcinoembryonic antigen (CEA).

37. The monospecific triabody according to claim 34, wherein the humanized monoclonal antibody is hMN-14.

38. The monospecific triabody according to claim 37, wherein each scFv comprises the V_H and the V_K regions of hMN-14.

39. The monospecific triabody according to claim 38, wherein each scFv comprises the amino acid sequence of SEQ ID NO:6.

40. An expression vector comprising a nucleotide sequence encoding the monospecific triabody of claim 34.

41. A host cell comprising the expression vector of claim 40.

42. A multivalent, monospecific binding protein comprising four binding sites having affinity for the same single target antigen (termed a monospecific tetrabody), wherein said binding sites are formed by the association of four single chain Fv (scFv) fragments, and wherein each scFv fragment comprises at least 2 variable domains derived from a humanized or human monoclonal antibody.

43. The monospecific tetrabody according to claim 42, wherein said monoclonal antibody is specific for a tumor-associated antigen.

44. The monospecific tetrabody according to claim 43, wherein said tumor-associated antigen is carcinoembryonic antigen (CEA).

45. The monospecific tetrabody according to claim 42, wherein the humanized monoclonal antibody is hMN-14.

46. The monospecific tetrabody according to claim 45, wherein each scFv comprises the V_H and the V_K regions of hMN-14.

47. The monospecific tetrabody according to claim 46, wherein each scFv further comprises an amino acid linker connecting the V_H and the V_K regions of hMN-14.

48. The monospecific tetrabody according to claim 47, wherein each scFv comprises the amino acid sequence of SEQ ID NO:8.

49. An expression vector comprising a nucleotide sequence encoding the monospecific tetrabody of claim 42.

50. A host cell comprising the expression vector of claim 49.

51. A method of diagnosing the presence of a tumor, said method comprising administering to a subject suspected of having a tumor a detectable amount of the binding protein of claim 9, and monitoring the subject to detect any binding of the binding protein to a tumor.

52. A method of treating a tumor, said method comprising administering to a subject in need thereof an effective amount of the binding protein of claim 19.

53. A method of diagnosing the presence of a tumor, said method comprising administering to a subject suspected of having a tumor a detectable amount of the binding protein of claim 1 in combination with a detectable moiety that is capable of binding to said binding protein, and monitoring the subject to detect any binding of the binding protein to a tumor.

54. A method of treating a tumor, said method comprising administering to a subject in need thereof an effective amount of the binding protein of claim 1 in combination with a therapeutic agent.

55. A method according to claim 54, wherein said therapeutic agent is selected from the group consisting of a chemotherapeutic drug, a toxin, external radiation, a brachytherapy radiation agent, a radiolabeled protein, an anticancer drug and an anticancer antibody.

56. A method of delivering one or more diagnostic agent, one or more therapeutic agent, or a combination of two or more thereof to a tumor, said method comprising administering to a subject in need thereof the binding protein of claim 8.

57. A kit for therapeutic and/or diagnostic use, said kit comprising at least one binding protein according to claim 8, and additional reagents, equipment, and instructions for use.

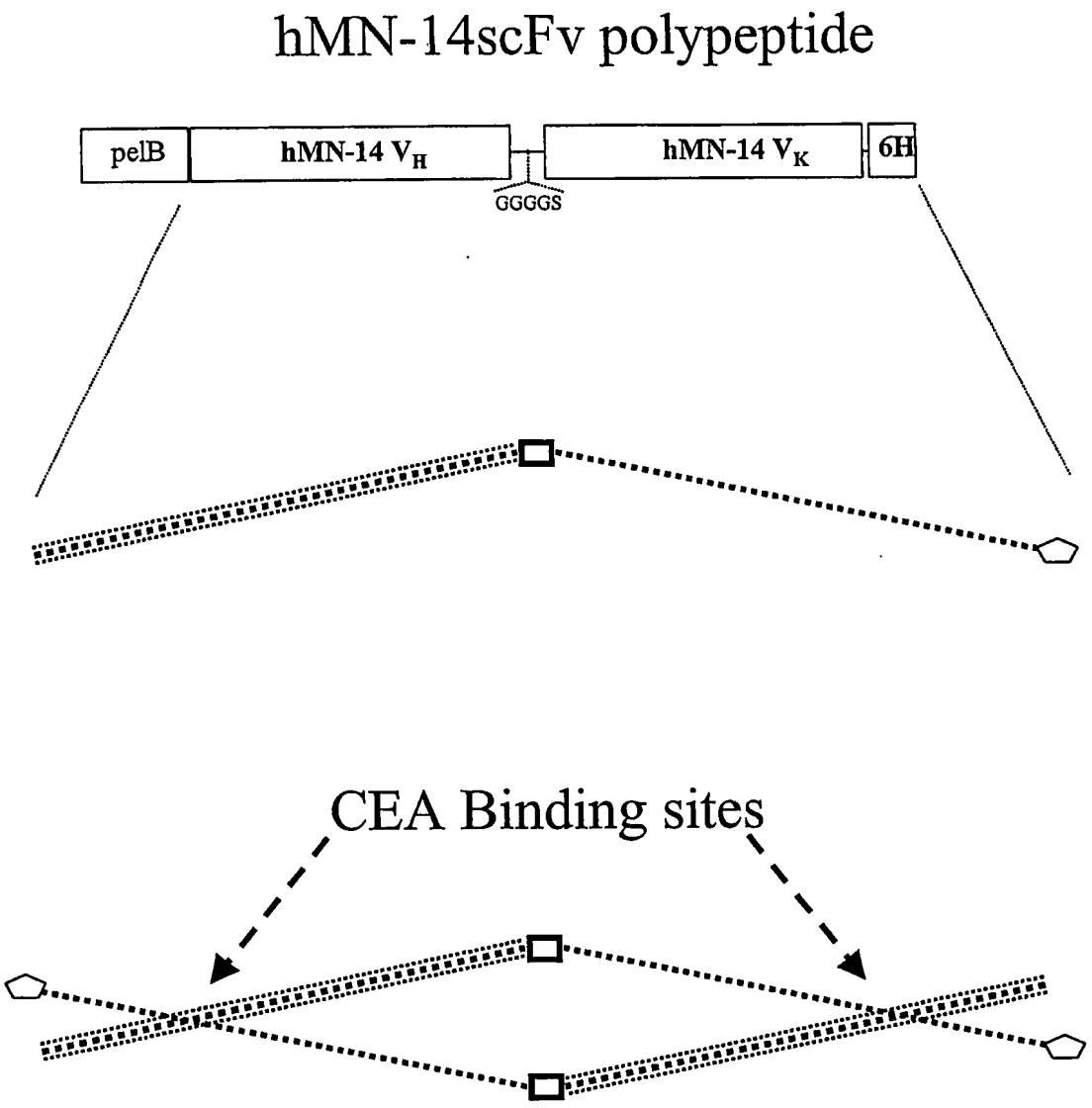

hMN-14scFv polypeptide
pelB
hMN-14 V_H
GGGGS
hMN-14 V_K
6H
CEA Binding sites
hMN-14 diabody

FIGURE 1

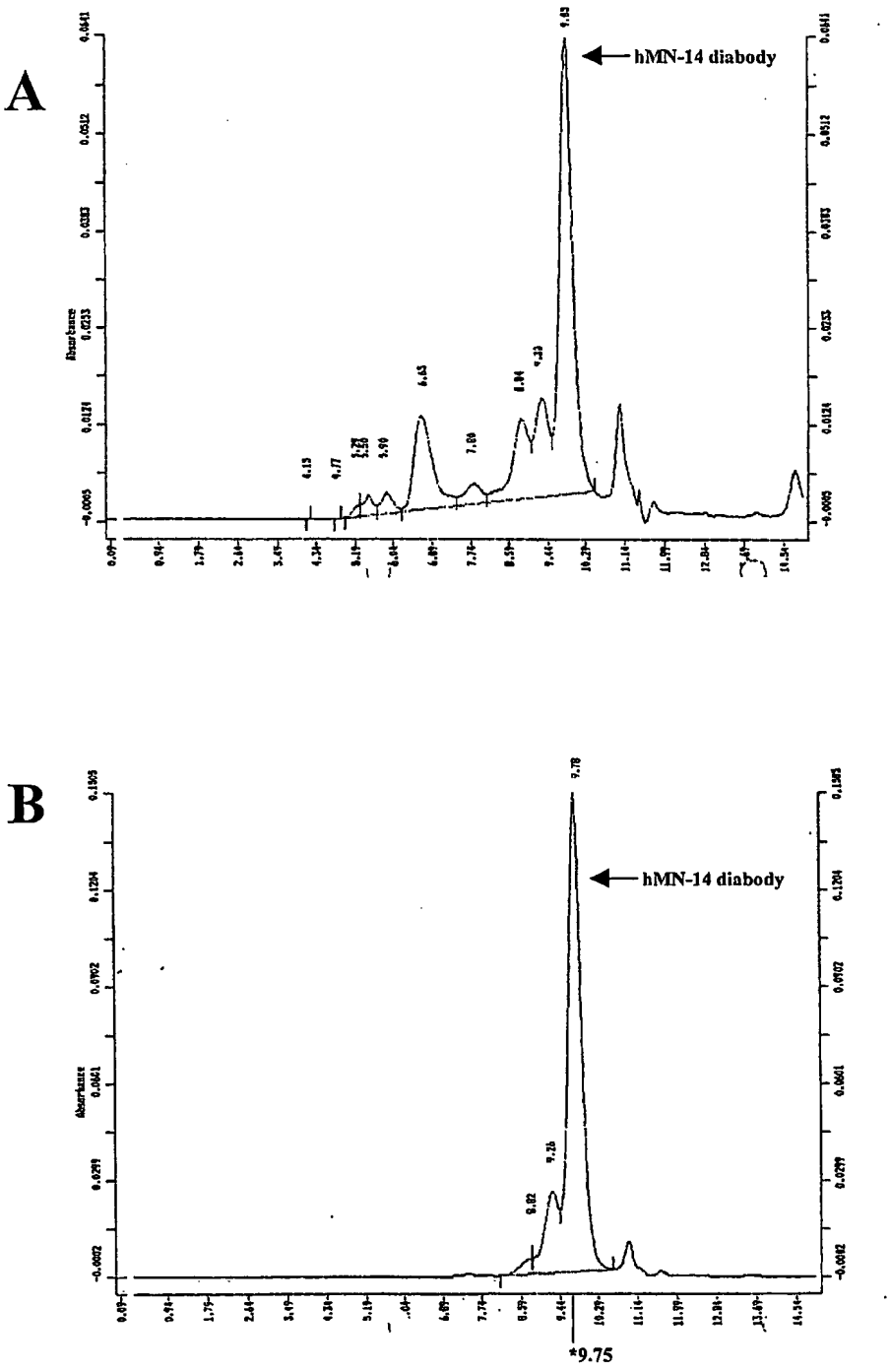


FIGURE 2

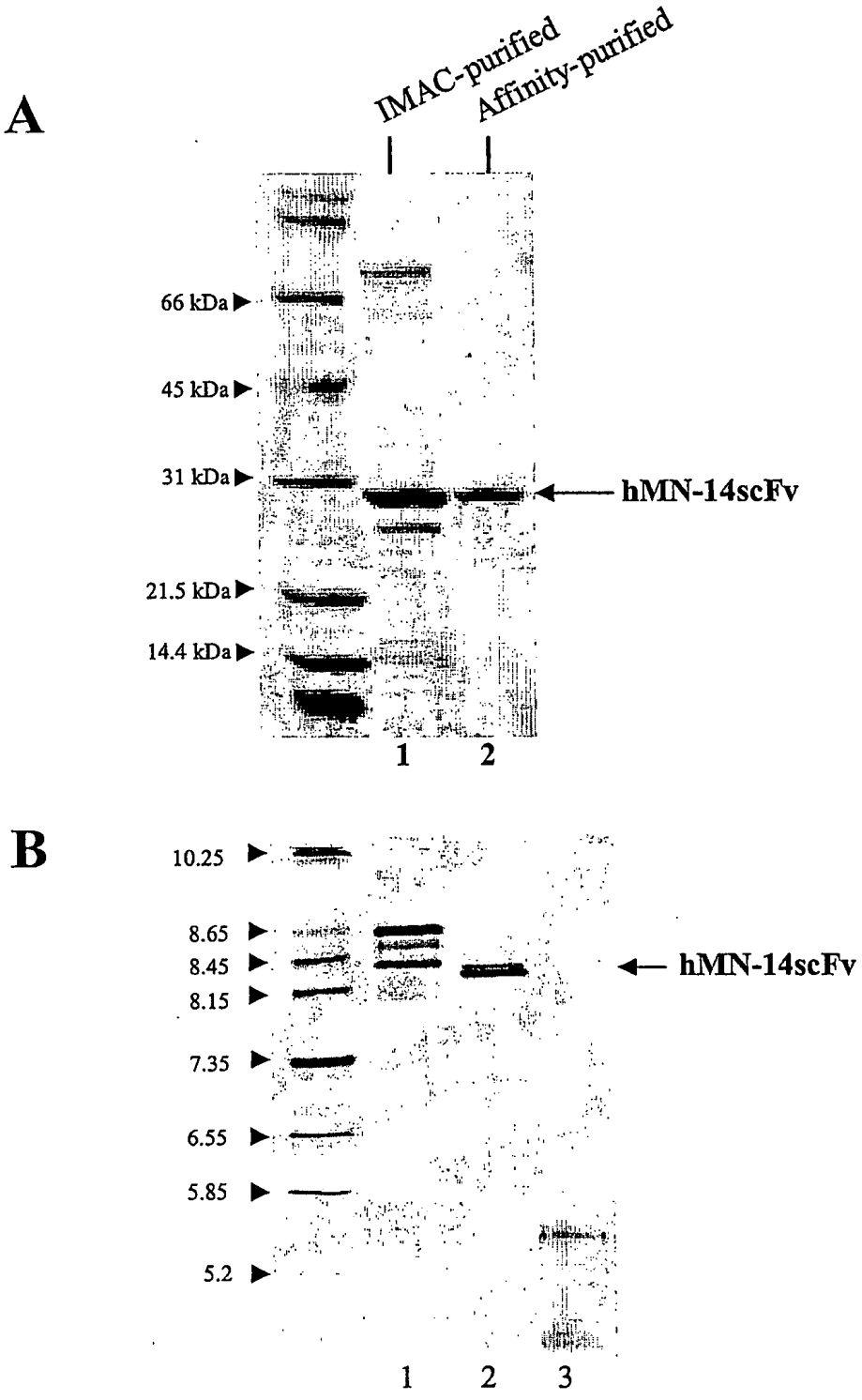

A
IMAC-purified
Affinity-purified
66 kDa
45 kDa
31 kDa
21.5 kDa
14.4 kDa
hMN-14scFv
1
2
B
10.25
8.65
8.45
8.15
7.35
6.55
5.85
5.2
hMN-14scFv
1
2
3

FIGURE 3

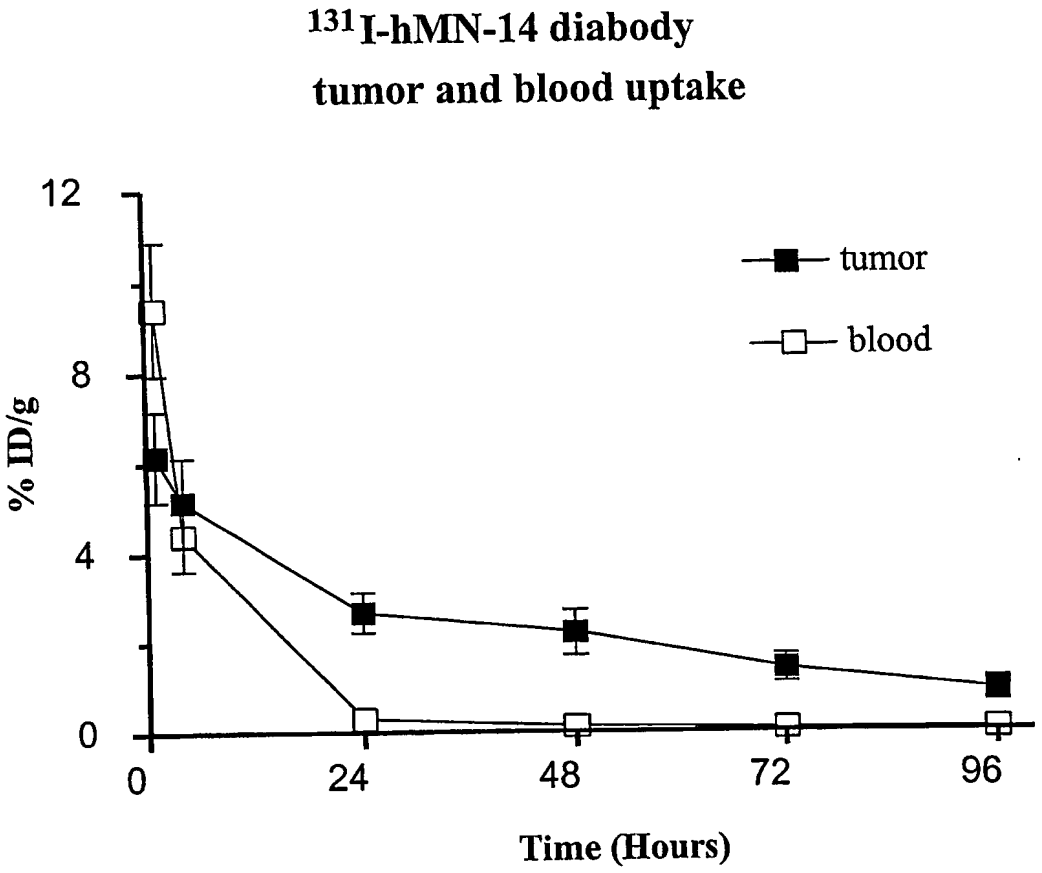

131I-hMN-14 diabody
tumor and blood uptake
12
8
4
0
% ID/g
tumor
blood
0
24
48
72
96
Time (Hours)

FIGURE 4

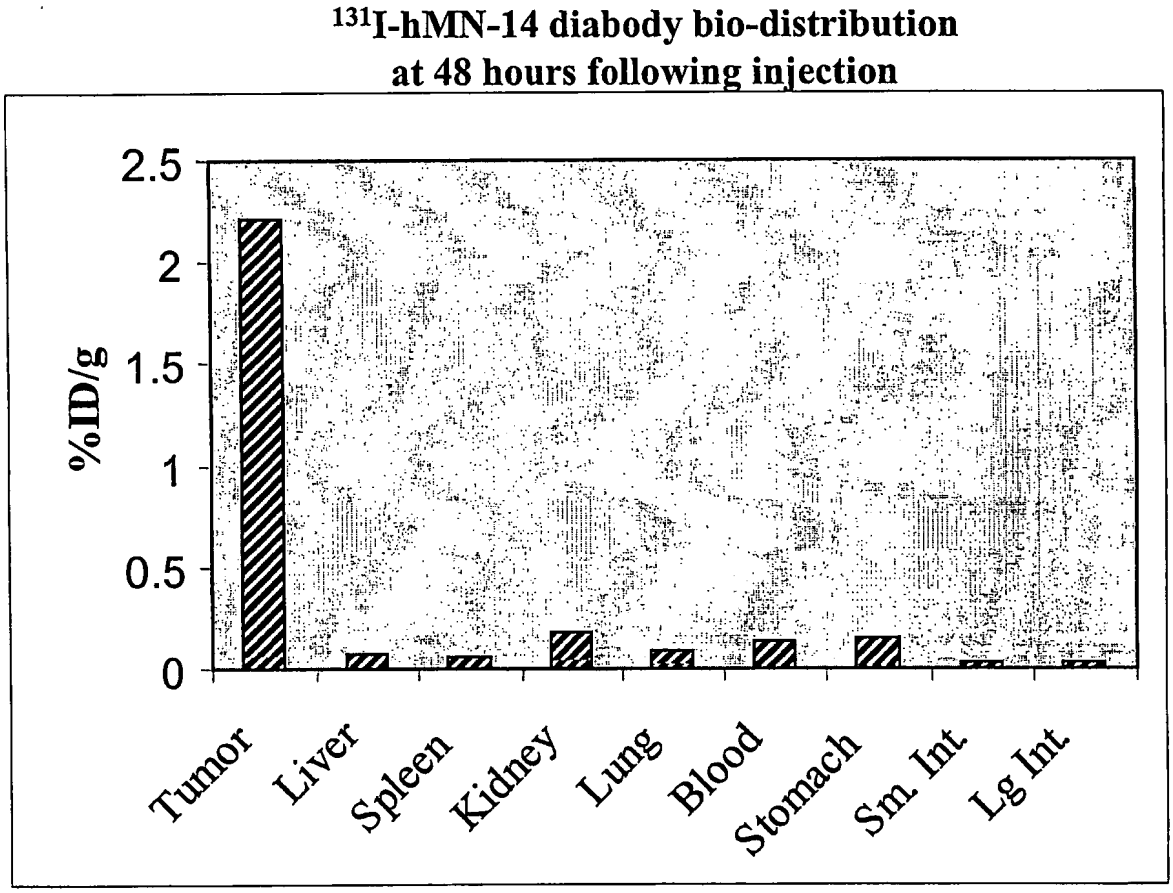
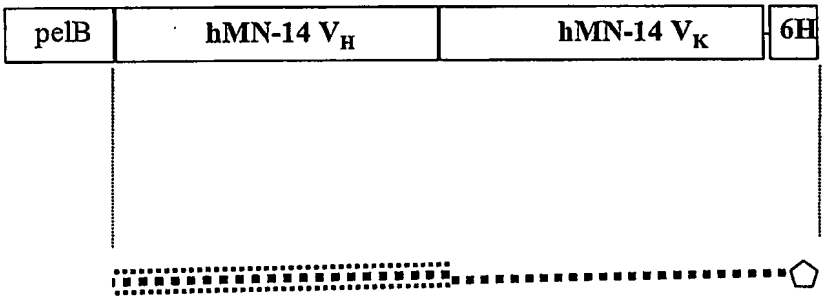
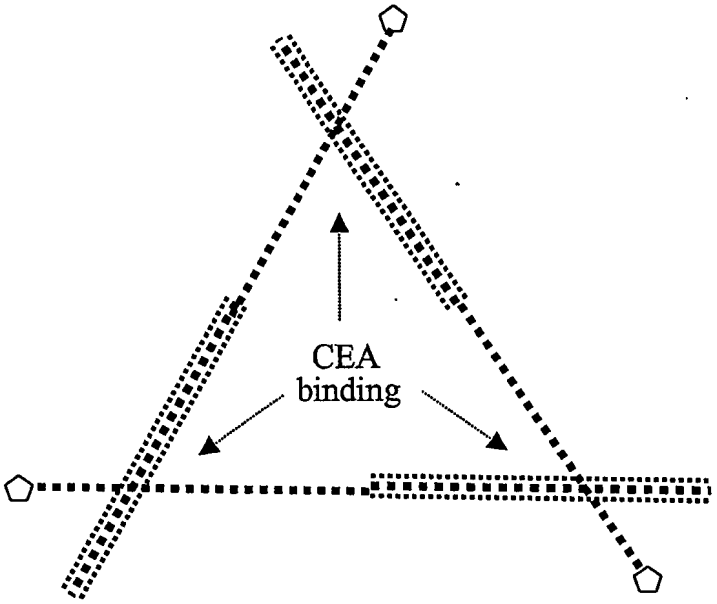

131I-hMN-14 diabody bio-distribution
at 48 hours following injection
%ID/g
2.5
2
1.5
1
0.5
0
Tumor
Liver
Spleen
Kidney
Lung
Blood
Stomach
Sm. Int.
Lg Int.

FIGURE 5

hMN-14scFv-0 polypeptide





hMN-14 triabody

FIGURE 6

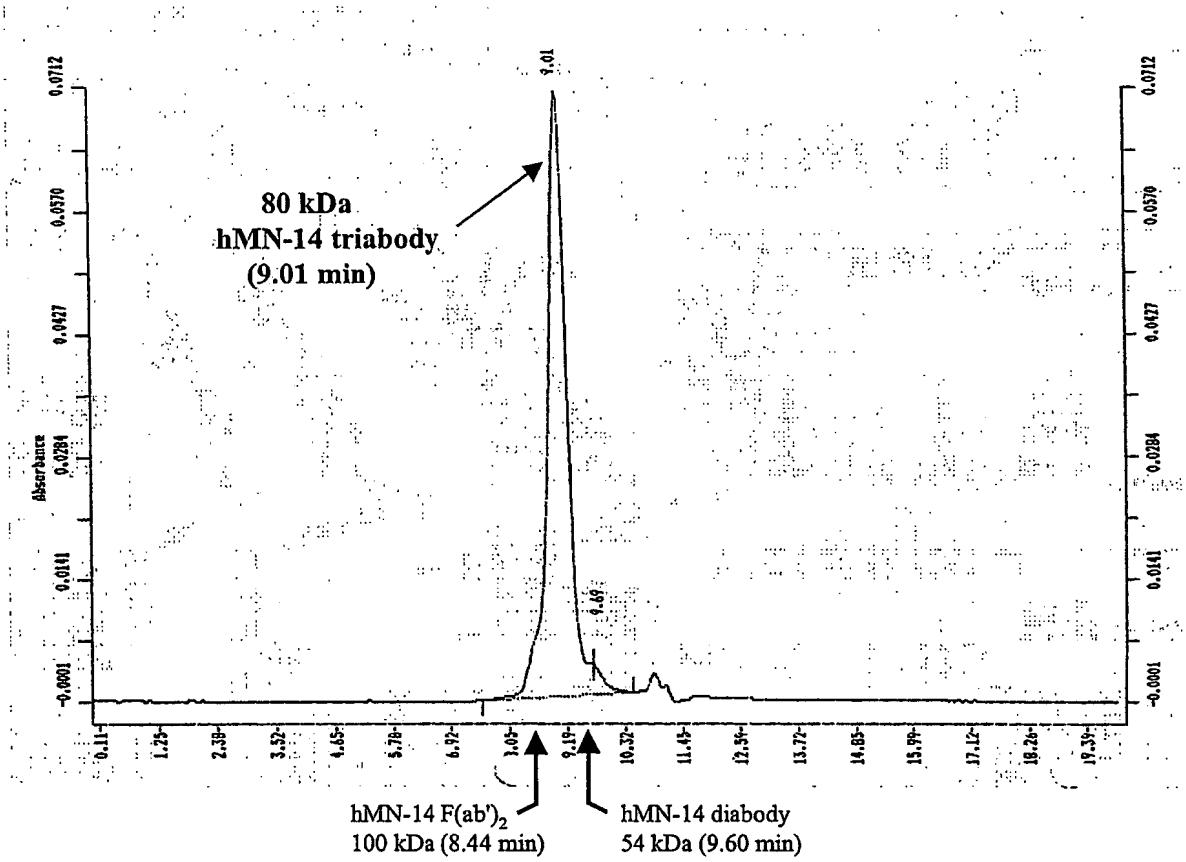

80 kDa
hMN-14 triabody
(9.01 min)
hMN-14 F(ab')2
100 kDa (8.44 min)
hMN-14 diabody
54 kDa (9.60 min)

FIGURE 7

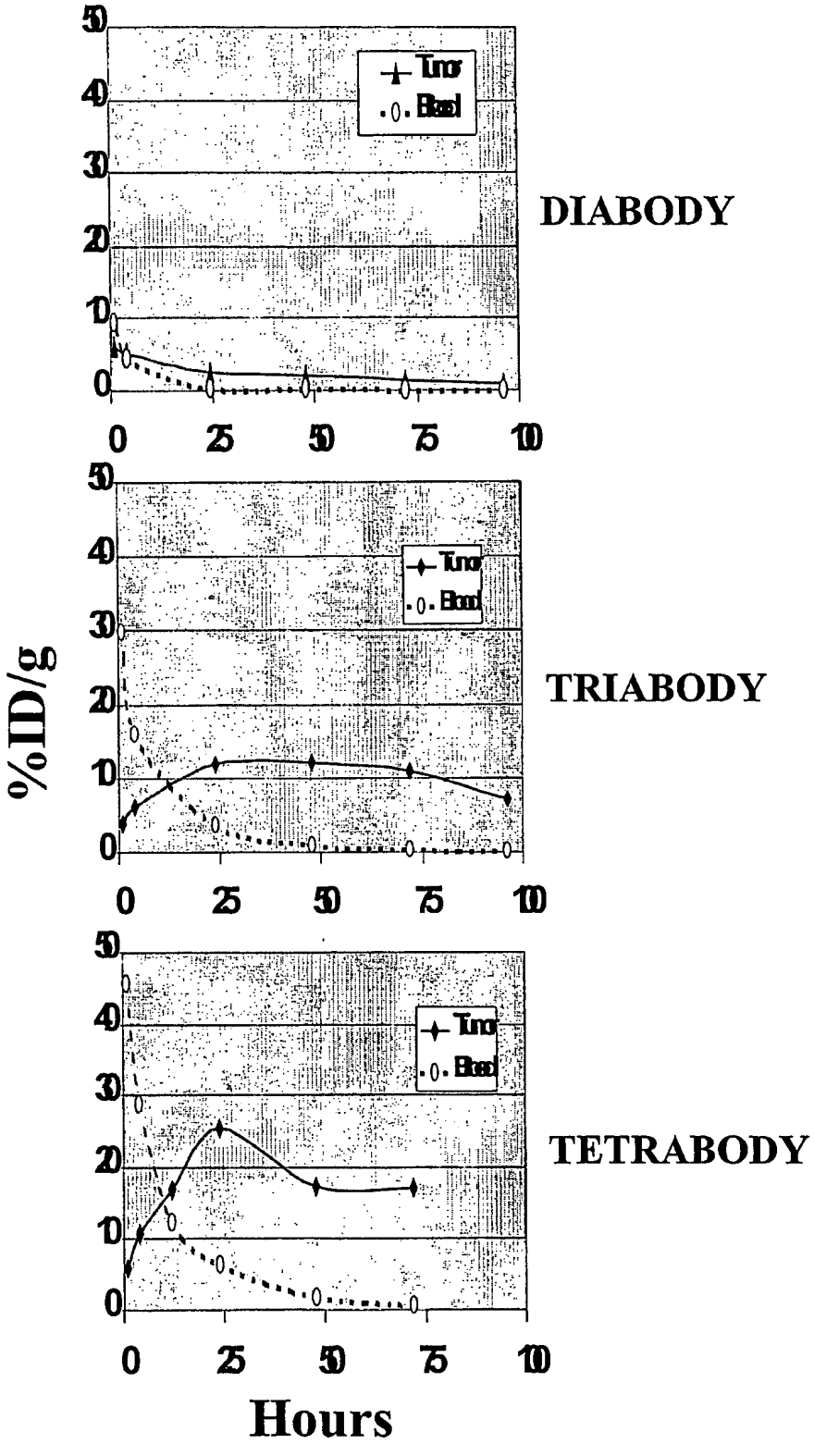
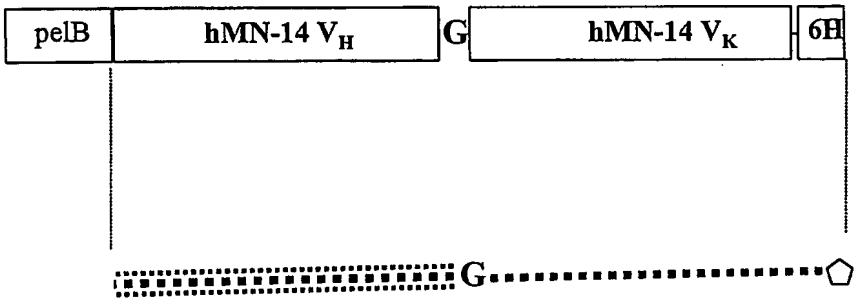

A
B
C
DIABODY
TRIABODY
TETRABODY
%ID/g
Hours
Tumor
Blood
0
10
20
30
40
50
0
25
50
75
100

FIGURE 8

hMN-14scFv-1G polypeptide



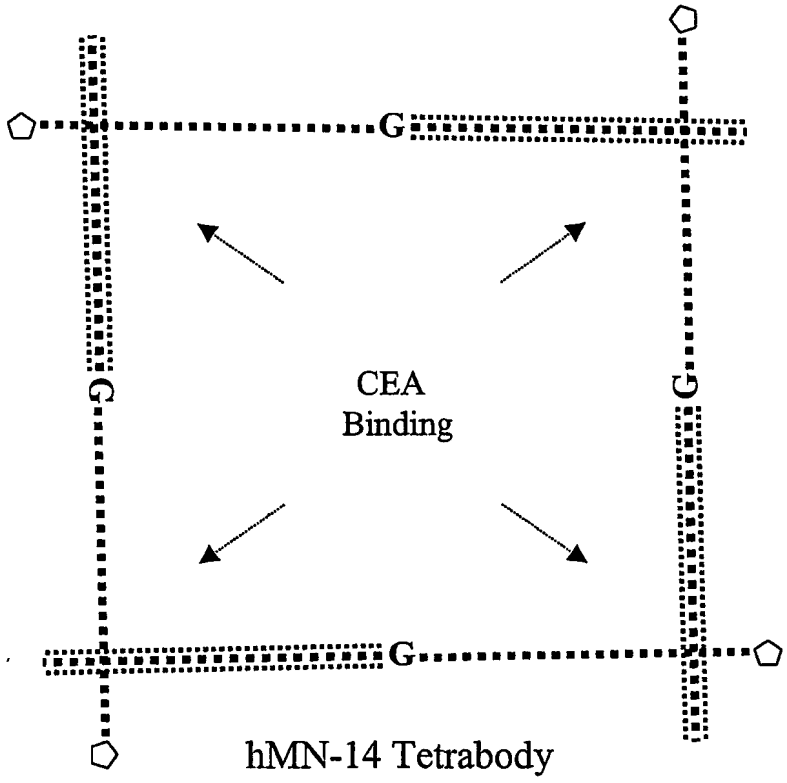
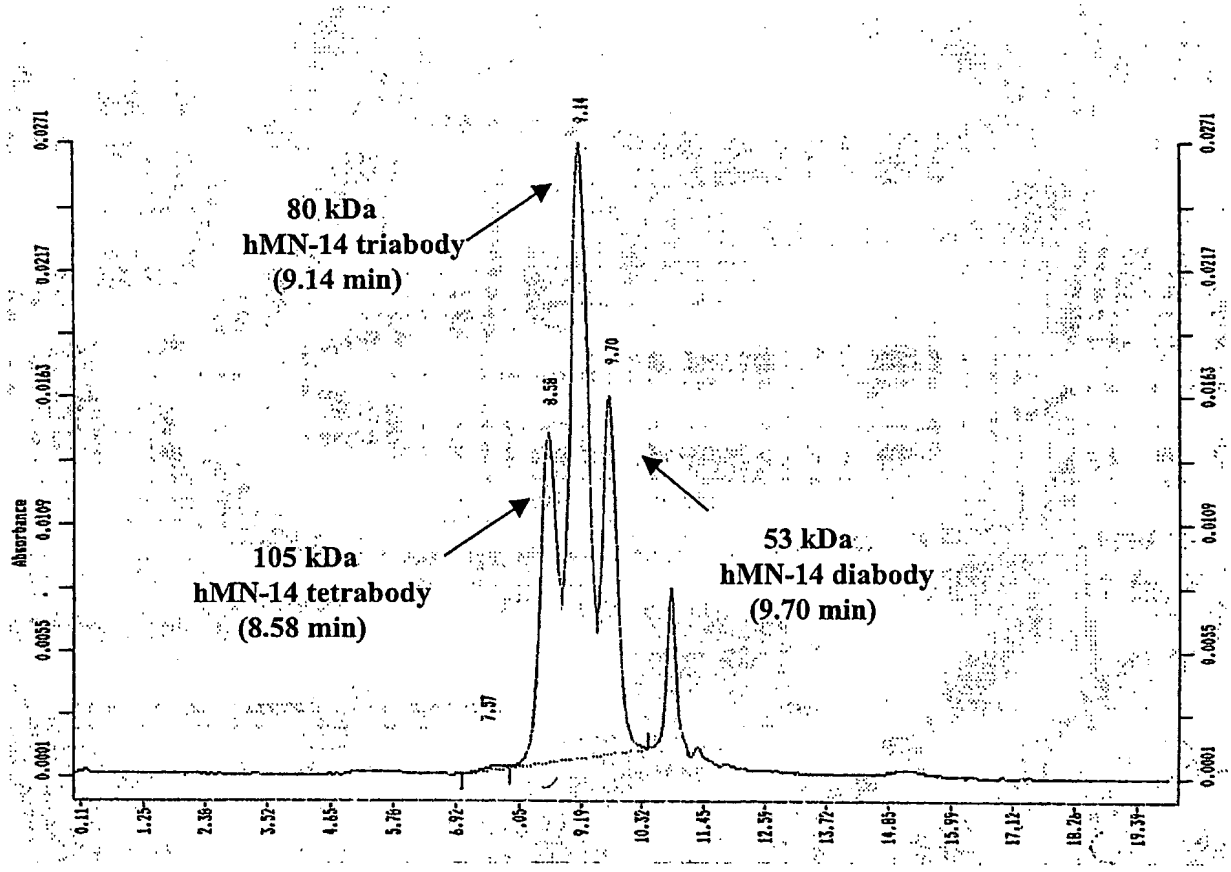


FIGURE 9


80 kDa
hMN-14 triabody
(9.14 min)
105 kDa
hMN-14 tetrabody
(8.58 min)
53 kDa
hMN-14 diabody
(9.70 min)
Absorbance
9.14
9.70
8.58
7.57

```
  1  ATG AAA TAC CTG CTG CCG ACC GCT GCT GCT GGT CTG CTG CTC CTC GCT
     Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala

 49  GCC CAG CCG GCG ATG GCC ATG GAG GTC CAA CTG GTG GAG AGC GGT GGA
     Ala Gln Pro Ala Met Ala Met Glu Val Gln Leu Val Glu Ser Gly Gly

 97  GGT GTT GTG CAA CCT GGC CGG TCC CTG CGC CTG TCC TGC TCC GCA TCT
     Gly Val Val Gln Pro Gly Arg Ser Leu Arg Leu Ser Cys Ser Ala Ser

145  GGC TTC GAT TTC ACC ACA TAT TGG ATG AGT TGG GTG AGA CAG GCA CCT
     Gly Phe Asp Phe Thr Thr Tyr Trp Met Ser Trp Val Arg Gln Ala Pro

193  GGA AAA GGT CTT GAG TGG ATT GGA GAA ATT CAT CCA GAT AGC AGT ACG
     Gly Lys Gly Leu Glu Trp Ile Gly Glu Ile His Pro Asp Ser Ser Thr

241  ATT AAC TAT GCG CCG TCT CTA AAG GAT AGA TTT ACA ATA TCG CGA GAC
     Ile Asn Tyr Ala Pro Ser Leu Lys Asp Arg Phe Thr Ile Ser Arg Asp

289  AAC GCC AAG AAC ACA TTG TTC CTG CAA ATG GAC AGC CTG AGA CCC GAA
     Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Asp Ser Leu Arg Pro Glu

337  GAC ACC GGG GTC TAT TTT TGT GCA AGC CTT TAC TTC GGC TTC CCC TGG
     Asp Thr Gly Val Tyr Phe Cys Ala Ser Leu Tyr Phe Gly Phe Pro Trp

385  TTT GCT TAT TGG GGC CAA GGG ACC CCG GTC ACC GTC TCC GGA GGC GGT
     Phe Ala Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Gly Gly Gly

433  GGA TCC GAC ATC CAG CTG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC
     Gly Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser

481  GTG GGT GAC AGA GTG ACC ATC ACC TGT AAG GCC AGT CAG GAT GTG GGT
     Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly

529  ACT TCT GTA GCC TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG
     Thr Ser Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu

577  CTG ATC TAC TGG ACA TCC ACC CGG CAC ACT GGT GTG CCA AGC AGA TTC
     Leu Ile Tyr Trp Thr Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe

625  AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC
     Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu

673  CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAA TAT AGC CTC TAT
     Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Leu Tyr

721  CGG TCG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT CTC GAG CAC
     Arg Ser Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Leu Glu His

769  CAC CAC CAC CAC CAC TGA (SEQ ID NO:1)
     His His His His His --- (SEQ ID NO:2)
```

Nucleic acid and deduced amino acid sequences of hMN-14-scF$_v$-L5

FIGURE 11

A) Deduced amino acid sequence of hMN-14V_H

EVQLVESGGG VVQPGRSLRL SCSASGFDFT TYWMSWVRQA PGKGLEWIGE IHPDSSTINY
APSLKDRFTI SRDNAKNTLF LQMDSLRPED TGVYFCASLY FGFPWFAYWG QGTPVTVS
(SEQ ID NO:3)

B) Deduced amino acid sequence of hMN-14V_K

DIQLTQSPSS LSASVGDRVT ITCKASQDVG TSVAWYQQKP GKAPKLLIYW TSTRHTGVPS
RFSGSGSGTD FTFTISSLQP EDIATYYCQQ YSLYRSFGQG TKVEIKRLEH HHHHH
(SEQ ID NO:4)

Deduced amino acid sequences of hMN-14-V_H and V_K

FIGURE 12

```
  1  ATG AAA TAC CTG CTG CCG ACC GCT GCT GCT GGT CTG CTG CTC CTC GCT
     Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala

 49  GCC CAG CCG GCG ATG GCC ATG GAG GTC CAA CTG GTG GAG AGC GGT GGA
     Ala Gln Pro Ala Met Ala Met Glu Val Gln Leu Val Glu Ser Gly Gly

 97  GGT GTT GTG CAA CCT GGC CGG TCC CTG CGC CTG TCC TGC TCC GCA TCT
     Gly Val Val Gln Pro Gly Arg Ser Leu Arg Leu Ser Cys Ser Ala Ser

145  GGC TTC GAT TTC ACC ACA TAT TGG ATG AGT TGG GTG AGA CAG GCA CCT
     Gly Phe Asp Phe Thr Thr Tyr Trp Met Ser Trp Val Arg Gln Ala Pro

193  GGA AAA GGT CTT GAG TGG ATT GGA GAA ATT CAT CCA GAT AGC AGT ACG
     Gly Lys Gly Leu Glu Trp Ile Gly Glu Ile His Pro Asp Ser Ser Thr

241  ATT AAC TAT GCG CCG TCT CTA AAG GAT AGA TTT ACA ATA TCG CGA GAC
     Ile Asn Tyr Ala Pro Ser Leu Lys Asp Arg Phe Thr Ile Ser Arg Asp

289  AAC GCC AAG AAC ACA TTG TTC CTG CAA ATG GAC AGC CTG AGA CCC GAA
     Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Asp Ser Leu Arg Pro Glu

337  GAC ACC GGG GTC TAT TTT TGT GCA AGC CTT TAC TTC GGC TTC CCC TGG
     Asp Thr Gly Val Tyr Phe Cys Ala Ser Leu Tyr Phe Gly Phe Pro Trp

385  TTT GCT TAT TGG GGC CAA GGG ACC CCG GTC ACC GTC TCC GAT ATC CAG
     Phe Ala Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Asp Ile Gln

433  CTG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG
     Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val

481  ACC ATC ACC TGT AAG GCC AGT CAG GAT GTG GGT ACT TCT GTA GCT TGG
     Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ser Val Ala Trp

529  TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC TGG ACA
     Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Trp Thr

577  TCC ACC CGG CAC ACT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT AGC
     Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser

625  GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG GAC ATC
     Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu Asp Ile

673  GCC ACC TAC TAC TGC CAG CAA TAT AGC CTC TAT CGG TCG TTC GGC CAA
     Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Leu Tyr Arg Ser Phe Gly Gln

721  GGG ACC AAG GTG GAA ATC AAA CGT CTC GAG CAC CAC CAC CAC CAC CAC
     Gly Thr Lys Val Glu Ile Lys Arg Leu Glu His His His His His His
```

(SEQ ID NO:5; nucleic acid)
(SEQ ID NO:6; amino acid)

Nucleic acid and deduced amino acid sequences of hMN-14-0

FIGURE 13

```
  1  ATG AAA TAC CTG CTG CCG ACC GCT GCT GCT GGT CTG CTG CTC CTC GCT
     Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala

 49  GCC CAG CCG GCG ATG GCC ATG GAG GTC CAA CTG GTG GAG AGC GGT GGA
     Ala Gln Pro Ala Met Ala Met Glu Val Gln Leu Val Glu Ser Gly Gly

 97  GGT GTT GTG CAA CCT GGC CGG TCC CTG CGC CTG TCC TGC TCC GCA TCT
     Gly Val Val Gln Pro Gly Arg Ser Leu Arg Leu Ser Cys Ser Ala Ser

145  GGC TTC GAT TTC ACC ACA TAT TGG ATG AGT TGG GTG AGA CAG GCA CCT
     Gly Phe Asp Phe Thr Thr Tyr Trp Met Ser Trp Val Arg Gln Ala Pro

193  GGA AAA GGT CTT GAG TGG ATT GGA GAA ATT CAT CCA GAT AGC AGT ACG
     Gly Lys Gly Leu Glu Trp Ile Gly Glu Ile His Pro Asp Ser Ser Thr

241  ATT AAC TAT GCG CCG TCT CTA AAG GAT AGA TTT ACA ATA TCG CGA GAC
     Ile Asn Tyr Ala Pro Ser Leu Lys Asp Arg Phe Thr Ile Ser Arg Asp

289  AAC GCC AAG AAC ACA TTG TTC CTG CAA ATG GAC AGC CTG AGA CCC GAA
     Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Asp Ser Leu Arg Pro Glu

337  GAC ACC GGG GTC TAT TTT TGT GCA AGC CTT TAC TTC GGC TTC CCC TGG
     Asp Thr Gly Val Tyr Phe Cys Ala Ser Leu Tyr Phe Gly Phe Pro Trp

385  TTT GCT TAT TGG GGC CAA GGG ACC CCG GTC ACC GTC TCC GGT GAT ATC
     Phe Ala Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Gly Asp Ile

433  CAG CTG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA
     Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg

481  GTG ACC ATC ACC TGT AAG GCC AGT CAG GAT GTG GGT ACT TCT GTA GCC
     Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ser Val Ala

529  TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC TGG
     Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Trp

577  ACA TCC ACC CGG CAC ACT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT
     Thr Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe Ser Gly Ser Gly

625  AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG GAC
     Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu Asp

673  ATC GCC ACC TAC TAC TGC CAG CAA TAT AGC CTC TAT CGG TCG TTC GGC
     Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Leu Tyr Arg Ser Phe Gly

721  CAA GGG ACC AAG GTG GAA ATC AAA CGT CTC GAG CAC CAC CAC CAC CAC
     Gln Gly Thr Lys Val Glu Ile Lys Arg Leu Glu His His His His His

769  CAC TGA (SEQ ID NO:7)
     His --- (SEQ ID NO:8)
```

Nucleic acid and deduced amino acid sequences of hMN-14-1G

FIGURE 14

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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60/404,919	22 August 2002 (22.08.2002)	US

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(71) Applicant and
(72) Inventor: **ROSSI, Edmund** [US/US]; c/o Immunomedics, Inc., 300 American Road, Morris Plains, NJ 07945 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CHANG, Chien-Hsing, Ken** [US/US]; c/o Immunomedics, Inc., 300 American Road, Morris Plains, NJ 07945 (US). **GOLDENBERG, David, M.** [US/US]; 330 Pleasant Valley Road, Mendham, NJ 07945 (US).

(74) Agents: **MAEBIUS, Stephen, B.** et al.; Foley & Lardner, Washington Harbour, 3000 K Street, N.W., Suite 500, Washington, DC 20007-5143 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
13 November 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/033654 A3

(54) Title: DIRECT TARGETING BINDING PROTEINS

(57) Abstract: The present invention relates to multivalent, monospecific binding proteins. These binding proteins comprise two or more binding sites, where each binding site specifically binds to the same type of target cell, and preferably with the same antigen on such a target cell. The present invention further relates to compositions of monospecific diabodies, triabodies, and tetrabodies, and to recombinant vectors useful for the expression of these functional binding proteins in a microbial host. Also provided are methods of using invention compositions in the treatment and/or diagnosis of tumors.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/32718

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/85, 5/12, 15/63; A61K 39/395; C07K 16/00
US CL : 530/387.3, 388.1, 388.85, 391.7; 435/320.1, 325; 424/133.1, 134.1, 135.1, 156.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.3, 388.1, 388.85, 391.7; 435/320.1, 325; 424/133.1, 134.1, 135.1, 156.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,693,762 A (QUEEN ET AL) 02 December 1997 (02/12/97), see entire document, especially abstract and column 1-2.	1-57
Y	US 6,121,424 A (WHITLOW ET AL) 19 September 2000 (19/09/00), see entire document, especially Figure 4B, 6A, 6B.	1-57
Y	PLUCKTHUN et al. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology. 1997, Vol.3, pages 83-105, especially figure 3.	1-57
X	US 6,096,289 A (GOLDENBERG) 01 August 2000 (01/08/00), see entire document,	1-12, 16-21, 25-33
---	especially column 15-16, 18, 19, 22.	---
Y		13-15, 22-24, 34-57
Y	US 5,837,242 A (HOLLIGER ET AL) 17 November 1998 (17/11/98), see entire document, especially Figure 1, column 8, 16, 22, 23, 26,	1-57

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
21 May 2003 (21.05.2003)

Date of mailing of the international search report
09 SEP 2003

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Janice Ford for

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INTERNATIONAL SEARCH REPORT

PCT/US02/32718

Continuation of B. FIELDS SEARCHED Item 3:
CAPLUS, MEDLINE, CANCERLIT, BIOSIS, WEST, USPATFUL
Search terms: scfv, diabody, triabody, tumor, CEA, label, MRI, hMN-14, inventors names.